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(A State University Established by the Government of Tamil Nadu)

KARAIKUDI – 630 003

Directorate of Distance Education

M.Sc. [Zoology]

IV - Semester

350 44

LAB IV: FISHERIES AND AQUACULTURE, ANIMAL BIOTECHNOLOGY, BIOPHYSICS, BIOSTATISTICS AND BIOINFORMATICS

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Phone: 0120-4078900 • Fax: 0120-4078999
Regd. Office: A-27, 2nd Floor, Mohan Co-operative Industrial Estate, New Delhi 1100 44
• Website: www.vikaspublishing.com • Email: helpline@vikaspublishing.com

Work Order No.AU/DDE/DE12-16/Printing of Course Material/2020 dated 28.02.2020, Copies - 500

SYLLABI-BOOK MAPPING TABLE

Lab IV: Fisheries and Aquaculture, Animal Biotechnology, Biophysics, Biostatistics and Bioinformatics

Syllabi

FISHERIES AND AQUACULTURE

1. Identification of commercially Important Fin Fishes, Shell Fishes, Molluscs, Lobsters and Seaweed.
2. Physical, Biochemical and Microbiological Methods to Examine Freshness of Fish.
3. Estimation of Protein, Lipid, Carbohydrate and Salt Content in Fish.
4. Determination of Stocking Density and Feed Assessment.
5. Method of Transportation of Seeds.
6. Modern Crafts and Gears.

ANIMAL BIOTECHNOLOGY

1. Isolation of Genomic DNA.
2. Estimation of DNA.
3. Demonstration of ELISA.
4. RAPD, RFLP (Demo).
5. Extraction and Purification of Plasmid DNA.
6. Spotter: Models of PCR, Southern Blotting.
7. Cloning Vectors – Images.

BIOPHYSICS, BIOSTATISTICS AND BIOINFORMATICS

1. Spectrophotometer, pH Meter, and Electrophoretic Unit as Spotters.
2. Construction of Graph and Bar Diagram using Biological Data.
3. Calculation of Mean, Median, Mode, Variance, Standard Deviation and Standard Error, and Chi-Square Test.
4. IN SILICO Analysis.

INTRODUCTION

NOTES

In biology, the term fish is most strictly used to describe any animal with a backbone that has gills throughout life and has limbs, if any, in the shape of fins. In fisheries, the term fish is used as a collective term and includes molluscs, crustaceans and any aquatic animal which is harvested. True fish observes the strict biological definition of a fish and are also referred to as finfish or fin fish to distinguish them from other aquatic life harvested in fisheries or aquaculture.

Aquaculture, also known as aquafarming, is the farming of fish, crustaceans, molluscs, aquatic plants, algae, and other organisms. Aquaculture involves cultivating freshwater and saltwater populations under controlled conditions, and can be contrasted with commercial fishing, which is the harvesting of wild fish. According to the Food and Agriculture Organization (FAO), aquaculture is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants.

Animal biotechnology is the use of science and genetic engineering to modify living organisms. Under the umbrella of genetic engineering exist other technologies, such as transgenics and cloning. Examples of animal biotechnology include creating transgenic animals, using gene knock out technology to make animals with a specific inactivated gene and producing nearly identical animals by somatic cell nuclear transfer (or cloning).

Biophysics is an interdisciplinary science that applies approaches and methods traditionally used in physics to study biological phenomena. The term biophysics was originally introduced by Karl Pearson in 1892. Biophysics covers all scales of biological organization, from molecular to organismic and populations.

Biostatistics (also known as biometry) are the development and application of statistical methods to a wide range of topics in biology. It encompasses the design of biological experiments, the collection and analysis of data from those experiments and the interpretation of the results.

Bioinformatics is an interdisciplinary field that develops methods and software tools for understanding biological data, in particular when the data sets are large and complex. Bioinformatics and computational biology involve the analysis of biological data, particularly DNA, RNA, and protein sequences.

This lab manual, *Fisheries and Aquaculture, Animal Biotechnology, Biophysics, Biostatistics and Bioinformatics*, explains about the fisheries and aquaculture, identification of commercially important fishes, methods to examine freshness of fish; estimation of protein, lipid, carbohydrate and salt content in fish; method of transportation of seeds, crafts and gears, isolation of genomic DNA, estimation of DNA, ELISA, RAPD, RFLP, extraction and purification of plasmid DNA, biophysics, biostatistics and bioinformatics.

FISHERIES AND AQUACULTURE

Definition of Aquaculture

It is method of rearing, breeding and harvesting of aquatic species (animals and plants). It helps in food production, restoration of threatened and endangered species population, increasing wild stock population, preparing of aquariums and fish cultures and habitat restoration.

Aquaculture depends upon the following:

1. Hydrobiological Features
2. Motive of Farming
3. Special Operational Techniques

Types of Aquaculture

- Mariculture – It is done with the help of seawater. It is a process of farming of marine organisms for food and other products like pharmaceuticals, jewellery, cosmetics, etc.
- Fish Farming – It is the most commonly used method in which selective breeding of fish in fresh water or may be in sea water. As it is a cheap source of protein, so highly exploited one.
- Algaculture – In this farming of species of algae is done which shares the plant and animal features.
- Integrated Multi-Trophic Aquaculture (IMTA) – In this two or more organisms are farmed together as different trophic levels are combined into one system to cater to various nutritional demand.
- Inland Pond Culture – Variety of aquatic species cultured in ponds with the method of pond to be built and situated at proper place, water level to be maintained along with the quality and quantity.
- Recirculating Systems – This method is environment friendly as very less amount of water is used by keeping fish in one and water treatment in different chambers. Evaporated water is being replaced with new water by accurate power supply in order to maintain the temperature, oxygen and other factors which can cause harm to fish.
- Open-Net Pen and Cage Systems – With the help of public water, fish in the pens, waste, chemicals, parasites and diseases are transferred as per the water environments.
- Flow-Through / Raceway – A very long unit is being stocked with the fish includes feeding stations enclosed with them. Waste being collected at the down of the unit is disposed of through water diverted from flowing water.

Benefits of Aquaculture – In terms of economic factors like alternative food source, alternative fuel source, increase of employment in the market and also helps in reducing sea food trade deficit. As far as environmental benefits are

concerned it acts as barrier against pollution with mollusc and seaweed, reduces fishing pressure on wild stock, low environmental impact, adequate water usage.

Importance of Aquaculture

- Conservation of biodiversity by slowing down the fishing activities on the wild stock.
- Sustainable use of sea resources by providing idea to replenish wild stock over the time.
- Health Benefit in terms of demand being increased as seafood are essential source of maintaining healthy diet and helps in fighting against diseases, like Cancer, Alzheimer, etc.
- It helps in reducing environmental disturbance.

Process of Aquaculture

1. Breeding
2. Pre-Growing
3. On-Growing
4. Harvesting and Packaging
5. Sales and Distribution

1. Identification of Commercially Important Fin Fishes, Shell Fishes, Molluscs, Lobsters and Seaweed.

Fish is composed of 70-80% water, 15-20% proteins, 1-13% fat, and 1% minerals. Fat, also known as lipids, contains important omega-3 fatty acids that provide health benefits by helping to lower cholesterol and blood pressure. Generally speaking, fish have an easier life in the water than land animals so their muscles do not work as hard.

Fish are generally divided into different categories depending on their ***family*** and ***species***, whether they are ***fresh*** or ***saltwater*** fish, by their body shape (if they are ***round***, ***flat***, or ***cartilaginous*** fish), by fat content, whether they are ***fatty*** or ***lean*** fish, and if they are ***cold-water*** or ***warm-water*** fish.

Families are large groups of fish, for example salmon, and the species include the Atlantic, King, or Coho varieties. The Salmon family also includes Trout and Char.

There are differences in flavour between salt water and freshwater fish. Saltwater fish, because of the salinity of their environment, produce sweet-tasting ***glycine*** and savoury ***glutamate amino acids*** that milder-tasting freshwater fish lack.

Round fish are symmetrical with identical colouring on both sides, while ***flat fish*** swim sideways, have asymmetrical eyes (eyes on one side of the head), and are darkly pigmented on their top side while white on the bottom. Cartilaginous fish includes shark, skate, and ray, containing no ossified bones.

Fat content determines the best method for cooking fish. Lean fish that have almost no fat content become dry when overcooked, while fattier fish are able to tolerate more heat without drying too quickly.

Warm-water fish are found along the Gulf Stream, Reefs, and Warm Seas like the Mediterranean. ***Cold-water fish*** are from the deep waters or colder areas of the oceans and have characteristics that include firm flesh and higher levels of fat.

Fish categories are grouped according to their families, and in some cases paired with varieties from other families that possess a similar structure, texture, and fat content.

For most fish the skeleton is made of ***bone,*** but some varieties, including shark, skate, and ray, are made of ***cartilage***. The skeletal structure, along with the size of the fish, determines how fish are filleted.

True Fish and Finfish

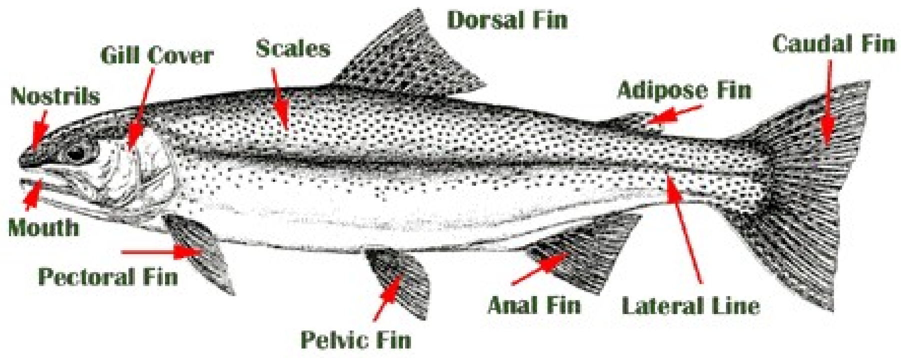
In biology, the term fish is most strictly used to describe any animal with a backbone that has gills throughout life and has limbs, if any, in the shape of fins. Many types of aquatic animals with common names ending in 'Fish' are not fish in this sense; examples include shellfish, cuttlefish, starfish, crayfish and jellyfish. In fisheries, the term fish is used as a collective term, and includes mollusks, crustaceans and any aquatic animal which is harvested.

The strict biological definition of a fish is sometimes called a true fish. True fish are also referred to as finfish or fin fish to distinguish them from other aquatic life harvested in fisheries or aquaculture.

Finfish Culture

Finfish means a bony fish generally referred to as true fish for example shark. It is assumed as an ancient occupation which is being there throughout many centuries.

Following figure illustrates the different types of fins in fish.



In order to have a better understanding regarding the fish fins, we should go through the following eight types of fish fins:

Dorsal Fins: These are flat thin part found on the back of the fish. It is made up of connective tissue, not bone. The main purpose of this fin is to maintain stability in the water and helps to control sudden movements. Mostly fishes have one dorsal fin but some fishes have two or three.

Example – Prussain cap have one dorsal fin, Sharks have two dorsal fins and Haddocks have three dorsal fins.

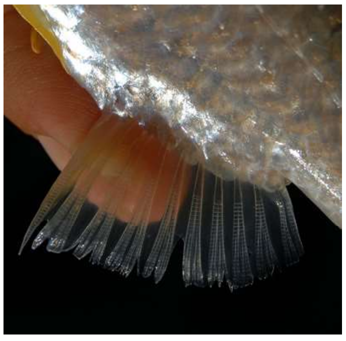


Dorsal Fin of a Shark

Caudal Fins: It is also known as tail fins. There are many types of caudal fins, such as

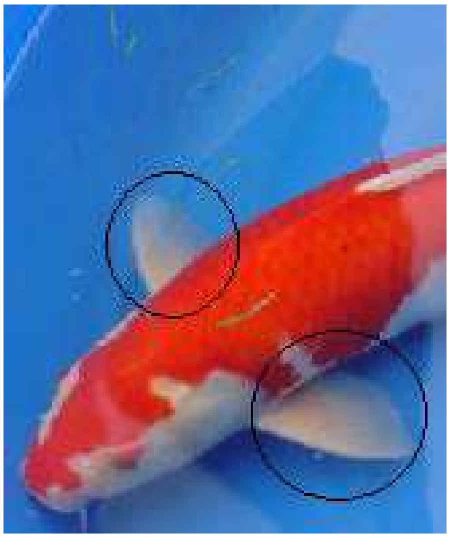
- **Heterocercal –** The vertebrae extend into the upper lobe of the tail thus making it longer. We can find this type of fins in shark.
- **Hypocercal –** The vertebrae extend into the lower lobe of the tail which makes it longer. It can be seen in Anaspida.
- **Protocercal –** The vertebrae extend to the tip of the tail, it is symmetrical but not expanded one. Best example is amphioxus.
- **Homocercal –** The vertebrae extend for a very short distance into the upper lobe of the fin.
- **Diphycercal –** The vertebrae extend to the tip of tail and also expanded with the symmetrical feature. Such as bichir, lungfish, lamprey and coelacanth.

Anal Fins: These are on the ventral (bottom) surface of the fish, behind the anus. Fish use these for stability while swimming.



Anal Fin

Pectoral Fins: These are located on each side of the fish, around where the head meets the body. These are often thought of as the 'Fish's Arms'. These type of fins can be seen in Mudskipper, Sea Robins and Flying Gurnards.



Pectoral Fins

Pelvic Fins: These are located on each side of the fish, near its bottom middle. The pelvic fins helps them in going upward and downward in the water, taking sharp turns and stopping quickly. It can take many positions along the ventral surface of the fish.

Let us see examples position wise:

- Abdominal Position seen in Minnows.
- Thoracic Position in Sunfish.
- Jugular Position in Burbot.



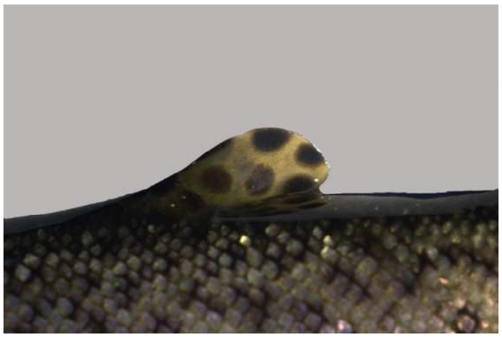
Pelvic Fins

Adipose Fins: These type of fins are located at the back behind the dorsal fin and just before the caudal fin. It helps fishes to be vital for detection of, and response

to changes such as touch, sound and any kind of change in pressure. Many readings have suggested that adipose fins can develop in two different ways.

- **Salmoniform-Type** – The fins develops from the larval-fin fold at the same time and in the same direct manner as the other median fins.
- **Characiform-Type** – The fin develops late after the larval-fin fold has diminished and the other median fins have developed.

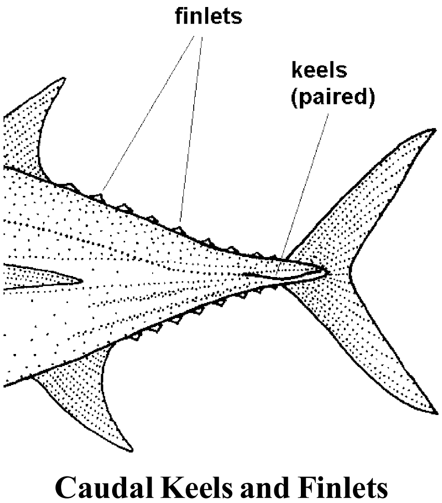
Examples – Myctophiformes, Aulopiformes, Stomiiformes, Salmoniformes, Osmeriformes, Characiformes, Siluriformes, Argentiniformes, etc.



Adipose Fin of a Trout

Caudal Keels: These are small horizontal fins just forward the caudal fin on each side of the fish. It is just like a keel of the ship usually composed of scutes, which provides stability and support to the 'Caudal Fin'. These type of fins are basically found in faster swimming fishes.

Finlets: are small fins that look like ridges along the top and bottom of the fish, behind the dorsal and anal fins. Examples are Bichirs, Tuna, Sauries.



Caudal Keels and Finlets

Commercially Important Fin Fish

Carp – It is of various species of oily freshwater fish from the family Cyprinidae, a very large group of fish native to Europe and Asia. While carp is consumed in

many parts of the world, they are generally considered as invasive species in parts of Africa, Australia and most of the United States.



Common Carp: Cyprinus carpio

In detail if we talk, is the Cypriniformes are grouped according to the common features they share together. The structure itself consists of a set of minute bones that originate from the first few vertebrae to develop in an embryonic ostariophysan. These bones grow to physically connect the auditory system, specifically the inner ear, to the swim bladder. The structure acts as an amplifier of sound waves that would otherwise be only slightly perceivable by the inner ear structure alone.



Goldfish

Carp have been important and old source of food fish to humans. Goldfish breeds and the domesticated common carp variety known as koi have been popular ornamental fishes. Several species of carp are considered invasive species in the United States and, worldwide, large sums of money are spent on carp control.

At least some species of carp are able to survive for months with practically no oxygen (for example under ice or in stagnant, scummy water) by metabolizing glycogen to form lactic acid which is then converted into ethanol and carbon dioxide. The ethanol diffuses into the surrounding water through the gills.



Carp

Common Species of Carp

Silver Carp



Grass Carp



Bighead Carp



Crucain Carp



The common carp, **Cyprinus carpio**, is originally from Central Europe. Several carp species were domesticated in East Asia. Carp that are originally from South Asia, for example Catla, Rohu and Mrigal are known as Indian Carp. Their hardiness and adaptability have allowed domesticated species to be propagated all around the world.

Catla



Rohu



Salmon

It is the common name for several species of ray-finned fish in the family Salmonidae. The fishes in this group are Trout, Char, Grayling and Whitefish. Salmon are native to North Atlantic region and Pacific Ocean.

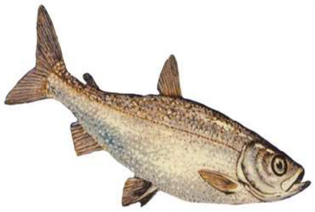
Trout



Char



Whitefish



Salmon hatch in fresh water, migrate to the ocean, then return to the fresh water for reproduction. Folklore is the fish return to the same spot where they hatched to eggs released.

Salmo



Common name is Atlantic salmon, scientific name is **Salmo salar.**

(Linnaeus, 1758)

Maximum Length – 150 cm

Common Length – 120 cm

Maximum Weight – 46.8 kg

Maximum Age – 13 years

Trophic Level – 4.4

Chinook Salmon



Common name is Chinook salmon, scientific name is **Oncorhynchus tshawytscha**.

(Walbaum, 1792)

Maximum Length – 150 cm

Common Length – 70 cm

Maximum Weight – 61.4 kg

Maximum Age – 9 years

Trophic Level – 4.4

Chum Salmon



Common name is Chum salmon, scientific name is **Oncorhynchus keta**. (Walbaum, 1792)

Maximum Length – 100 cm

Common Length – 58 cm

Maximum Weight – 15.9 kg

Maximum Age – 7 years

Trophic Level – 3.5

Masu Salmon



Common name is Masu salmon, scientific name is **Oncorhynchus masou**. (Brevoort, 1856)

Maximum Length – 79 cm

Common Length – 71 cm

Maximum Weight – 10.0 kg

Maximum Age – 3 years

Trophic Level – 3.6

Salmon fishes lays eggs in freshwater streams typically at high latitudes. The eggs hatch into alevin or sac fry. The fry quickly develop into parr with camouflaging vertical stripes. It stray for 6 months to 3 years in their natal stream before becoming smolts, which are differentiated by their bright, silvery colour with scales that are

easily rubbed off. Only 10% of all salmon eggs are estimated to survive to this stage.



Eggs in different stages of development. At times, only a few cells grow on top of the yolk, in the lower right, the blood vessels surround the yolk and in the upper left, the black eyes are visible, even the little lens.



Salmon fry hatching—the baby has grown around the remains of the yolk—visible are the arteries spinning around the yolk and small oil drops, also the gut, the spine, the main caudal blood vessel, the bladder, and the arcs of the gills.

The smolt body chemistry changes, allowing them to live in salt water. Few of the species of salmon remain in fresh water throughout their life cycle, the majority of them migrate on a regular basis to feed or reproduce.

Tilapia

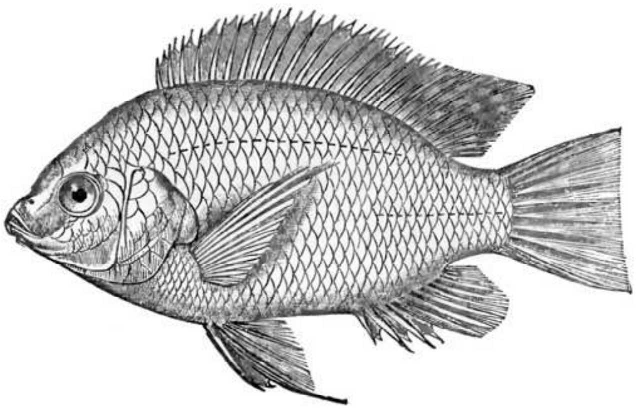
Tilapia are mainly freshwater fish inhabiting shallow streams, ponds, rivers, and lakes, and less commonly found living in brackish water. Historically, they have been of major importance in artisanal fishing in Africa, and they are of increasing importance in aquaculture and aquaponics. They can become a problematic invasive species in new warm water habitats such as Australia, whether deliberately or accidentally introduced, but generally not in temperate climates due to their inability to survive in cold water.

Features of Tilapia

- They have laterally compressed, deep bodies.
- Their lower pharyngeal bones are fused into a single tooth-bearing structure.
- Complex set of muscles allows the upper and lower pharyngeal bones to be used as a second set of jaws for processing food.
- Their mouths are protrusible, usually bordered with wide and often swollen lips.
- The jaws have conical teeth.
- It can exist or adapt to wide range of conditions.
- They are mouth-brooding species, means they carry the fertilized eggs and young fish in their mouths for several days after the yolk sac is absorbed.

Commercial Species

Nile Tilapia



Scientific Name – **Oreochromis niloticus** (Linnaeus, 1758)

Maximum Length – 60 cm

Maximum Weight – 4.324 kg

Maximum Age – 9 years

Trophic Level – 2.0

Blue Tilapia



Scientific Name – **Oreochromis aureus** (Steindachner, 1864)

Maximum Length – 45.7 cm

Common Length – 16 cm

Maximum Weight – 2.010 kg

Trophic Level – 2.1

Mozambique Tilapia



Scientific Name – **Oreochromis mossambicus** (Peters, 1852)

Maximum Length – 39 cm

Common Length – 35 cm

Maximum Weight – 1.130 kg

Maximum Age – 11 years

Trophic Level – 2.**0**

Unlike carnivorous fish, tilapia can feed on algae or any plant-based food. This reduces the cost of tilapia farming, reduces fishing pressure on prey species, and avoids concentrating toxins that accumulate at higher levels of the food chain.

Due to their large size, rapid growth, and palatability, tilapia cichlids are the focus of major farming efforts, specifically various species of Oreochromis, Sarotherodon, and Coptodon (all were formerly in the namesake genus Tilapia). Like other large fish, they are a good source of protein and popular among artisanal and commercial fisheries.

Commercially grown tilapia are almost exclusively male. This is typically done by adding male sex hormone in the food to the tilapia fry, causing any potential female tilapia to change sex to male. It can also be achieved through hybridization of certain tilapia species or the use of so-called 'Supermales' that have homozygous male sex chromosomes (resulting in all their offspring receiving a male sex chromosome and thus becoming males). Males are preferred because they grow much faster than females. Additionally, because tilapia are prolific breeders, the presence of female tilapia results in rapidly increasing populations of small fish, rather than a stable population of harvest-size animals.

NOTES

Other methods of tilapia population control are polyculture, with predators farmed alongside tilapia or hybridization with other species.

Catfish

They are a diverse group of ray-finned fish. Famous for prominent barbels which looks like a cat's whiskers. They range according to their size and habitat from the three largest species alive as follows:-

The Mekong Giant Catfish



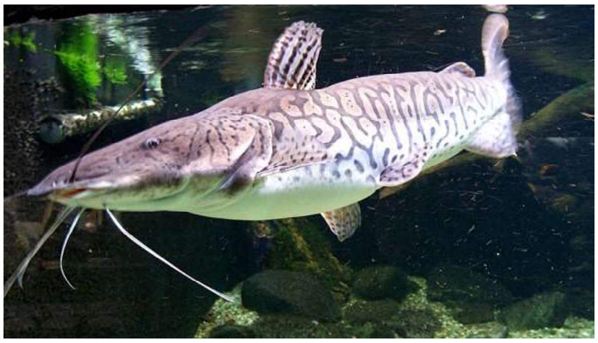
It is a large endangered species of catfish in the shark catfish family. It is basically from Southeast Asia.

The Wels Catfish



It is a large species of catfish native to wide areas of central, southern and Eastern Europe. It is a freshwater fish with broad, flat head and wide mouth. Wels catfish can live for at least fifty years.

The Piraiba



It is a largest species of catfish family name Pimelodidate, which is native to Amazon and Orinoco River basins, at South America.

The type of extant catfish species live inland or in coastal waters of every continent excluding Antarctica. They inhabited in all continents at one time or another. Most of the time they diverse in tropical South America, Asia and Africa, with one family nearer to North America and one family in Europe. Half of them species live in the Americas.

Some of them are represented with eight families like hypogean which live underground with another three families. For example, Phreatobius cisternarum, remain underground. Species like Ariidae, Plotosidae, Aspredinidae and Bagridae are found in salt water.

Physical Features

- Most of the catfish are bottom feeders.
- Due to heavy bony head and reduced gas bladder they usually sink rather than floating.
- Generally they have cylindrical body with a flattened ventrum to allow for benthic feeding.
- Some of them have a mouth that can expand to a large size.
- They generally feed through suction or directly gulping instead of biting and cutting prey.
- They may have up to four pair of barbels: nasal, maxillary (on each side of mouth), two pair of chin barbels.
- Catfish barbels always comes in pairs.
- With the help of chemosensor which is being found across their entire bodies, they can touch, taste and smell any chemical in present in the water.
- The primary role in the orientation and location of food is being done by the gustation present only in the catfish.
- They do not have scales, which make them naked.

Black Bullhead



They have the ability to thrive in waters which are low in oxygen, brackfish, turboid or very warm. It comes in three different colors like black, brown and yellow.

Walking Catfish



Channel Catfish



It has four pair of barbels.

Kryptopterus Vitreolus (Glass Catfish)



They have transparent bodies lacking both scales and pigments. All the Internal organs are located near the head.

Shellfishes

Shellfish dwell in water and have a shell or shell-like exterior. They can be divided into two groups: Crustaceans and Molluscs. Crustaceans include Shrimp, Crayfish, Crab, and Lobster, While Clams, Scallops, Oysters, and Mussels are examples of Molluscs.

Molluscs

Molluscs are the largest marine phylum lives in freshwater and terrestrial habitats.

Cephalopod molluscs – These are among the most neurologically advanced of all invertebrates. Like Squid, Cuttlefish and Octopuses.

The Gastropods – It comprises of 80% of the total classified species like Snails and Slugs.

Important Characteristics

1. They are mantle with a significant cavity which is used for breathing and excretion.
2. Presence of radula (a rasping tongue with chitinous teeth)
3. Structure of nervous system.

Squid

It is a sea creature with a long body and ten arms situated around the mouth, or this animal eaten as food. They are found both in coastal and oceanic waters. Squids may be swift swimmers or part of drifting sea life.



Squids have elongated bodies, large eyes, eight arms and two tentacles. They have distinct head, bilateral symmetry and a mantle.

Lifespan: Squids usually live from 3 to 5 years. Scientist are used to call this as a quick life history "r-section" with r signifying extreme reproduction and growth.

Three Hearts: Squid have three hearts: two branchial hearts and one systemic heart. The branchial hearts pump blood to the gills, where oxygen is taken up. Blood then flows to the systemic heart, where it is pumped to the rest of the body.

They possess chromatophores, specialized structures on their skin which contain pigment and allow them to change color rapidly.

Gills: They use oxygen from seawater for respiration. The seawater enters the mantle through the opening near the head, and passes over the gills. Oxygen diffuses

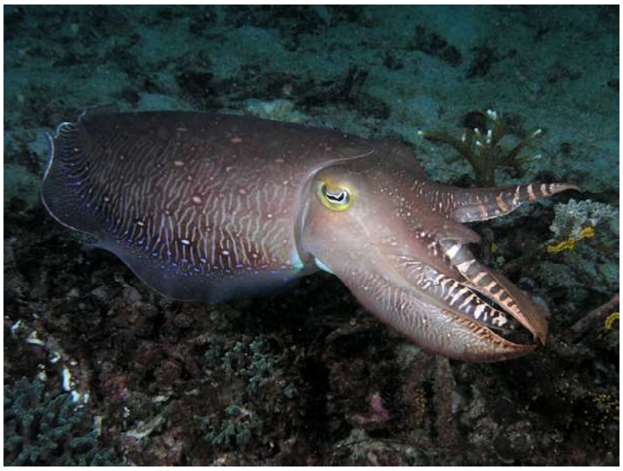
from the water into the blood, and is transported to the gill hearts by a network of many blood vessels.

Blood: Its blood is blue in colour, due to copper- containing compound called haemocyanin.

NOTES

Cuttlefishes

Cuttlefish or cuttles are marine molluscs of the order Sepiida. They belong to the class Cephalopoda, which also includes squid, octopuses, and nautiluses. Cuttlefish have a unique internal shell, the cuttlebone, which is used for control of buoyancy.



Lifespan: Cuttlefish have a short life span, but they grow quickly. They may only live one or two years, but some species can grow up to about 23 lbs (10.5 kg).

The skin of cuttlefish changes color rapidly using elastic pigment sacs called chromatophores, in order to evade predators. Cephalopods such as cuttlefish often use use adaptive camouflage to blend in with their surroundings.

Octopus

The octopus is a soft-bodied, eight-limbed mollusc of the order Octopoda. Around 300 species are recognised, and the order is grouped within the class Cephalopoda with squids, cuttlefish, and nautiloids.



Octopuses are sea animals famous for their rounded bodies, bulging eyes, and eight long arms. They live in all the world's oceans but are especially abundant in warm, tropical waters. Octopuses, like their cousin, the squid, are often considered "Monsters of the Ddeep," though some species, or types, occupy relatively shallow waters.

Most of the octopuses stay along the ocean's floor, although some species are pelagic, which means they live near the water's surface. Other octopus species live in deep, dark waters, rising from below at dawn and dusk to search for food. Crabs, shrimps, and lobsters rank among their favorite foods, though some can attack larger prey, like sharks. The octopus performs its famous backward swim by blasting water through a muscular tube on the body called a siphon. Octopuses also crawl along the ocean's floor, tucking their arms into small openings to search for food. Seals, whales, and large fish prey on octopuses.

Gastropoda

The class is made up of the snails, which have a shell into which the animal can generally withdraw, and the slugs, which are snails whose shells have been reduced to an internal fragment or completely lost in the course of evolution.



They are among the few groups of animals who have become successful in all three major habitats- the ocean, fresh waters, and land. The shells of some species are used as ornaments or in making jewellery.

Size Range and Diversity of Structure

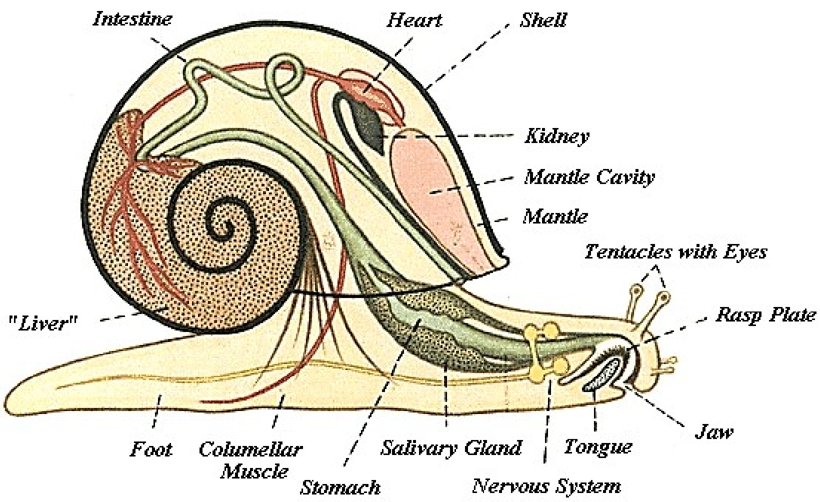
Some of the adult marine snails like Homalogyra and forest-litter snails like Stenophylis and Punctum are less than 1 millimetre in diameter. On the other hand the largest land snail – the African Achatina which forms a shell approx. 20 centimetres long.

Largest snail **Parenteroxenos doglieli** lives as a parasite in the body cavity of a sea cucumber which grows almost 130 centimetres in size.

NOTES



Anatomy



Snails are distinguished by an anatomical process known as torsion where the visceral mass of the animal rotates 180° to one side during development, such that the anus is situated more or less above the head. This process is unrelated to the coiling of the shell, which is a separate phenomenon. Torsion is present in all gastropods, but the opisthobranch gastropods are secondarily de-torted to various degrees.

Torsion occurs in two mechanistic stages. The first is muscular and the second is mutagenetic. The effects of torsion are primarily physiological - the organism develops an asymmetrical nature with the majority of growth occurring on the left side.

At the time of retracting into the shell, firstly posterior end would get pulled in followed by anterior. As a defensive purpose the front can be retracted more easily.

Gastropods typically have a well-defined head with two or four sensory tentacles with eyes, and a ventral foot. The foremost division of the foot is called

the **propodium**. Its function is to push away sediment as the snail crawls. The larval shell of a gastropod is called a protoconch.

Lobsters

Lobsters are a family of large marine crustaceans. All are marine and bottom-dwelling and most are nocturnal. They hunt for dead animals but also eat live fish, small mollusks and other bottom-dwelling invertebrates, and seaweed. Some of the species like true and spiny lobsters are commercially important food for human.



The American lobster (Homarus americanus) is the largest crustacenas.

Body

The lobster has a rigid, segmented body covering exoskeleton and five pairs of legs, one or more pairs of which are often modified into pincers with the chela on one side usually larger than that on the other. Lobsters have compound eyes on movable stalks, two pairs of long antennae, and several pairs of swimming legs (swimmerets) on the elongated abdomen. A flipperlike muscular tail is used for swimming; flexure of the tail and abdomen propel the animal backward.

Lobster anatomy includes two main body parts – the cephalothorax and the abdomen. The cephalothorax fuses the head and the thorax, both of which are covered by a chitinous carapace. The lobster's head bears antennae, antennules, mandibles, the first and second maxillae. The head also bears the (usually stalked) compound eyes. Because lobsters live in murky environments at the bottom of the ocean, they mostly use their antennae as sensors. The lobster eye has a reflective structure above a convex retina. In contrast, most complex eyes use refractive ray concentrators (lenses) and a concave retina. The lobster's thorax is composed of maxillipeds, appendages that function primarily as mouthparts, and pereipods, appendages that serve for walking and for gathering food. The abdomen includes pleopods (also known as swimmerets), used for swimming as well as the tail fan, composed of uropods and the telson.

Lobsters like snails and spiders, have blue blood due to hemocyanin present in their body. They also possess a green hepatopancreas called the tomalley by chefs, which functions as the animal's liver and pancreas.

Ecology

Lobsters live in all oceans, bottoms like sandy, rocky or muddy. Generally, they live singly on crevices or in burrows under rocks. They are omnivores and eat live prey such as fish, molluscs, other crustaceans, worms and some plant life.

In addition, they are 25-50 cm long and move slowly by walking on the sea floor. At the time of flee, they swim backward quickly by curling and uncurling their abdomens. They swim at the speed of 5m/s (11 mph) which is called as the cardioid escape reaction.

Lobsters are caught using baited one-way traps with a color-coded marker buoy to mark cages.

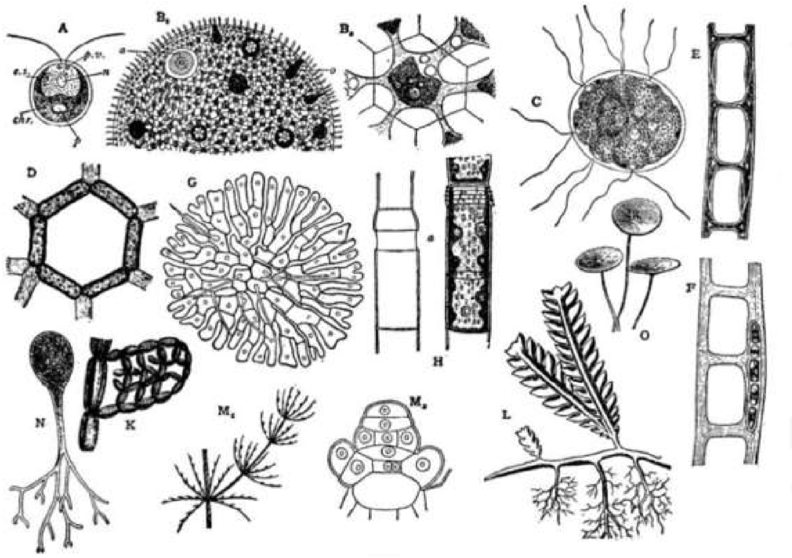
Lobster is fished in water between 2 and 900 metres (1 and 500 fathoms), although some lobsters live at 3,700 metres (2,000 fathoms). Cages are of plastic-coated galvanized steel or wood. A lobster fisher may tend as many as 2,000 traps. In a year 200, owing to overfishing and high demand, lobster aquaculture expanded.

Since 2008, no lobster aquaculture operation had achieved commercial success, mainly because of lobsters' tendency towards cannibalism and the slow growth of the species.

Commercially Important Seaweed

Seaweed refers to innumerable species of marine plants and algae that grows in the ocean, rivers, lakes and other water bodies. Seaweeds like phytoplankton which are microscopic lives suspended in the water column and thus gives the base for most of the marine food chains. There are thousands of species of seaweed which connects to both flowering plants submerged in the ocean, for example eelgrass and to larger marine algae.

Chlorophyta – Green Algae



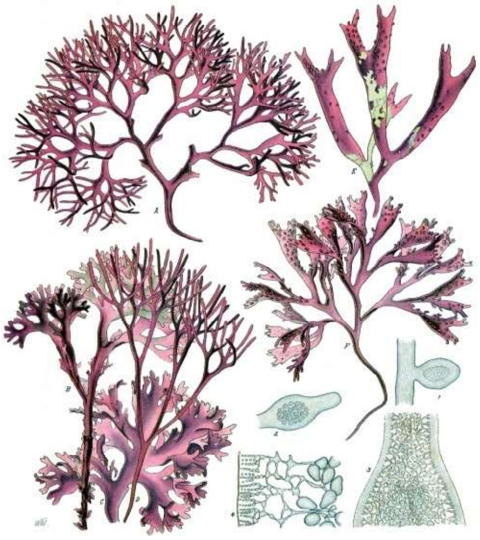
Phaeophyceae – Brown Algae



Chrysophyceae – Gold Algae (Mostly Found in Fresh Water)



Rhodophyta – Red Algae



NOTES

Anatomy

It looks like non-arboreal terrestrial plants which is as follows:

Thallus – A green shoot or twig

Lamina – A flattened structure like leaf

Sorus – Cluster

Pneumatocyst – A flotation assisting organ on the blade

Kelp – Flotation assisting organ between the lamina and stipe

Stipe – Structure like stem

Holdfast – Basal structure

Haptera – Finger-like extension

Ecology

Seaweed are being dominated by two environmental factors such as

- Seawater and light required to support photosynthesis
- Attachment point means seaweed mostly inhabits near shore waters
- Few of them which do not live attached to sea floor but float freely.

Farming

It is known as the process of cultivating and harvesting seaweed. It is being harvested throughout the world as a food source and also an export commodity for production of agar and carrageenan products.

Food

It is consumed across the world like East Asia – Japan, China, Korea, Taiwan, Southeast Asia – Brunel, Singapore, Thailand, Burma, Cambodia, Vietnam, Indonesia, Phillippines and Malaysia, South Africa, Southwest England etc.

Examples

Caulerpa – Green – Submerged



Fucus – Brown – In Intertidial Zones on Rocky Shares



Gracilaria – Red – Cultivated for food



Laminaria – Brown – Another name is kelps- cultivated for food



2. PHYSICAL, BIOCHEMICAL AND MICROBIOLOGICAL METHODS TO EXAMINE FRESHNESS OF FISH.

For fish and fishery products, freshness is one of the utmost principal attributes of fish quality, which makes a major contribution to the quality of fish and fishery products. It is well-known that the internal and intrinsic characteristics, such as fragile muscle tissue and activity of endogenous protease and inappropriate handling methods and storage conditions can be prone to resulting in physical, chemical, biochemical, and microbial changes and contaminations in fish, thus affecting its freshness quality.

In order to provide fish and fish products with premium quality, reliable methods and techniques for determination and evaluation of the freshness quality of fish are required. These can be methods based on biochemical and instrumental measurements, methods using refractive index or spoilage and freshness indices, and techniques based on sensor technology, such as colorimetric sensor array, semiconducting metal-oxide sensor array (Hammond *et al.*, 2002), gas sensor, or electronic nose. On the other hand, spectroscopy techniques are also widely used such as Near Infrared Reflectance (NIR) spectroscopy, mid-infrared reflectance (MIR) spectroscopy, Electrochemical Impedance Spectroscopy (EIS), and front-face fluorescence spectroscopy.

Methods for Evaluation of Fish Freshness

As we all know that freshness is the important factor of superior quality fish. Adaption of effective and correct methods for the examination of freshness of fish is very much necessary in research and industry for which methods are discussed below.

Physical Examination of Freshness of Fish

Sensory Evaluation

It is a method in which characteristics of food is analyzed by the senses of sight, smell and taste related to color, odor and texture of food. In this quality control can be divided into discriminative and descriptive test. Discriminative testing is used to determine if a difference exists between samples (triangle test, ranking test). Descriptive tests are used to determine the nature and intensity of the differences (profiling and quality tests). The subjective test is an affective test based on a measure of preference or acceptance.

Quality Index Method

The Quality Index Method (QIM) originally developed by the Tasmanian Food Research Unit (Bremner *et al.*, 1985) is used for fresh and frozen Cod, Herring and Saithe.

NOTES

Quality Assessment Scheme used to Identify the Quality Index Demerit Score (Larsen *et al.* 1992)

Quality Parameter	Character	Score (Ice/Seawater)
General Appearance	Skin	0 Bright, Shining 1 Bright 2 Dull
	Bloodspot on Gill Cover	0 None 1 Small, 10-30% 2 Big, 30-50% 3 Very Big, 50-100%
	Stiffness	0 Stiff, in *rigor mortis* 1 Elastic 2 Firm 3 Soft
	Belly	0 Firm 1 Soft 2 Belly Burst
	Smell	0 Fresh, Seaweed/Metallic 1 Neutral 2 Musty/Sour 3 Stale Meat/Rancid
Eyes	Clarity	0 Clear 1 Cloudy
	Shape	0 Normal 1 Plain 2 Sunken
Gills	Colour	0 Characteristic, Red 1 Faded, Discoloured
	Smell	0 Fresh, Seaweed/Metallic 1 Neutral 2 Sweaty/Slightly Rancid 3 Sour Stink/Stale, Rancid
Sum of Scores		(min. 0 and max. 20)

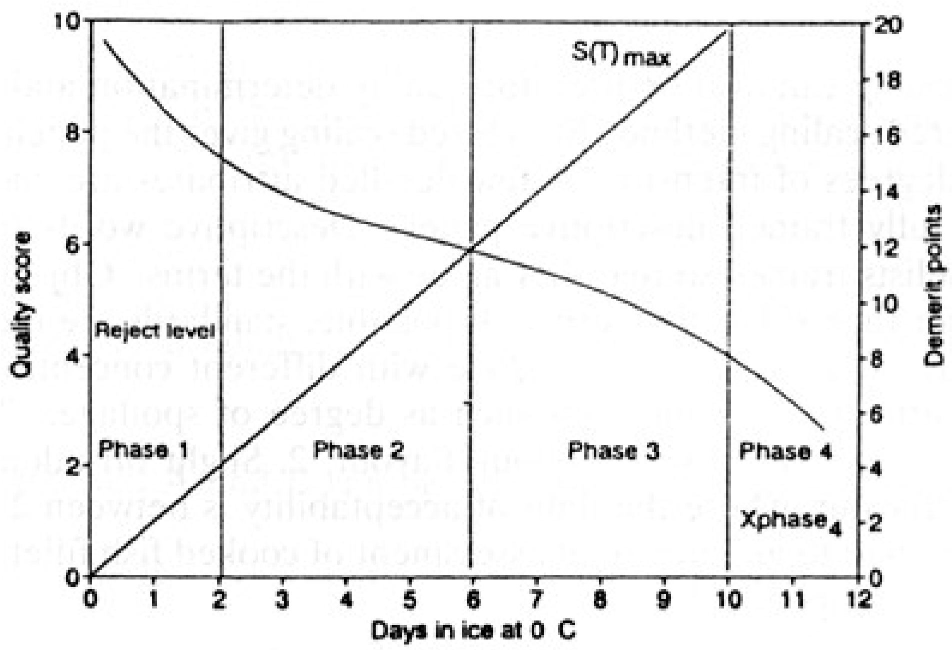
QIM is based on the significant sensory parameters for raw fish when using many parameters and a score system from 0 to 4 demerit points (Jonsdottir, 1992).

QIM is using a practical rating system, in which the fish is inspected and the fitting demerit point is recorded. The scores for all the characteristics are then summed to give an overall sensory score, the so-called quality index.

QIM gives scores of zero for very fresh fish while increasingly larger totals result as fish deteriorate. The description of evaluation of each parameter is written in a guideline. For example, 0 demerit point for the appearance of the skin on herring means very bright skin only experienced in freshly caught herring. The appearance of the skin in a later state of decay turns less bright and dull and gives 2 demerit points. Most of the parameters chosen are equal to many other schemes. After the literal description, the scores are ranked for each description for all the

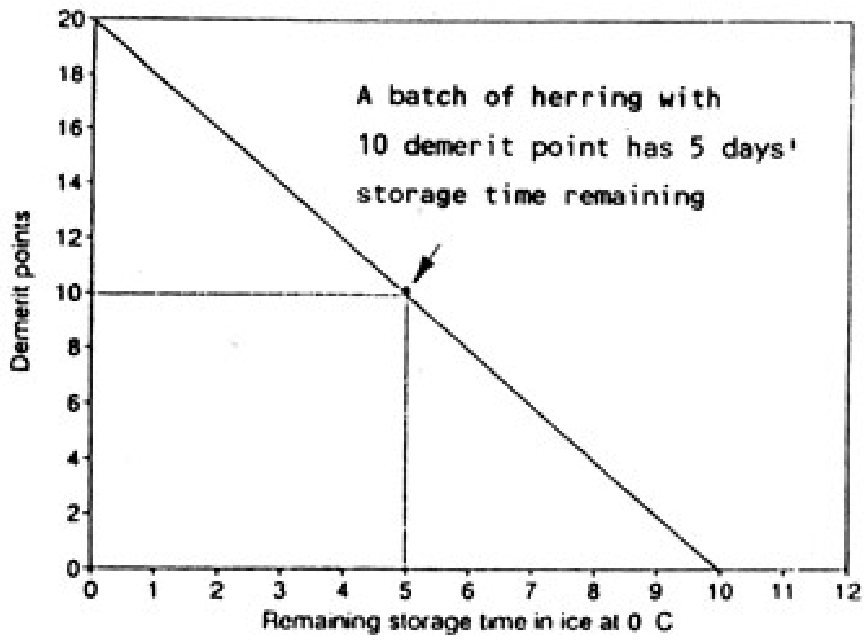
parameters, giving scores 0-1, 0-2, 0-3 or 0-4. Parameters with less importance are given lower scores. The individual scores never exceed 4, so no parameter can excessively unbalance the score.

There is a linear correlation between the sensory quality expressed as a demerit score and storage life on ice, which makes it possible to predict remaining storage life on ice. The theoretical demerit curve has a fixed point at (0,0) and its maximum has to be fixed as the point where the fish has been rejected by sensory evaluation of, for example, the cooked product or otherwise determined as the maximum keeping time. Using cooked evaluation the two parallel sensory tests demand an experienced sensory panel even though this is only required while developing the scheme, and later on it will not be necessary to assess cooked fish in order to predict the remaining shelf life. QIM does not follow the traditionally accepted S-curve pattern for deterioration of chilled fish during storage. The aim is a straight line which makes it possible to distinguish between fish at the start of the plateau phase and fish near the end of the plateau phase.



Combination of sensory curves for raw S(T) and cooked fish

When a batch of fish in above figure reaches a sum of demerit points of 10, the remaining keeping time in ice will be 5 days. To predict remaining shelf life, the theoretical curve can be converted as shown below.



A curve to predict the storage time remaining for herring stored in ice or sea water at 0°C

Structured Scaling

Descriptive testing can also be used for quality determination and shelf life studies applying a structured scaling method. Structured scaling gives the panellist an actual scale showing several degrees of intensity. A few detailed attributes are chosen often based on work from a fully trained descriptive panel. This can easily be done with different concentrations of salt but might be more difficult with conditions such as degree of spoilage. The simplest method can be 1. No off-odour/flavour, 2. Slight off-odour/flavour and 3. Severe off-odour/flavour, where the limit of acceptability is between 2 and 3.

Triangle Test

The most used discriminative test in sensory analysis of fish is the triangle test (ISO standard 4120 1983), which indicates whether or not a detectable difference exists between two samples. The assessors receive three coded samples, are told that two of the samples are identical and one is different, and are asked to identify the odd sample.

The number of correct identifications is compared to the number expected by use of a statistical table, e.g., if the number of responses is 12, there must be 9 correct responses to achieve a significant answer (1% level).

Triangle tests are useful in determining, for example, if ingredient substitution gives a detectable difference in a product. Triangle tests are often used when selecting assessors to a taste panel. Equal numbers of the six possible combinations are prepared and served to the panel members. They must be served randomly, preferably as duplicates. The number of panel members should be no less than 12.

Example of Score Sheet: Triangle Test

TRIANGLE TEST	
Name:	Date:
Type of sample:	
Two of these three samples are identical, the third is different. Examine the samples from left to right and circle the number of the test sample which is different. It is essential you make a choice (guess if no difference is apparent).	
Test sample No.:	
Describe the difference:	

Ranking

In a ranking exercise, a number of samples are presented to the taste panel. To arrange them in order according to the degree to which they exhibit some specified characteristics, for example, downward concentration of salt. Usually ranking can be done more quickly and with less training than evaluation by other methods. Thus ranking is often used for preliminary screening. The method gives no individual differences among samples and it is not suited for sessions where many criteria have to be judged simultaneously.

Profiling

Descriptive testing can be very simple and used for assessment of a single attribute of texture, flavour and appearance. Methods of descriptive analysis which can be used to generate a complete description of the fish product have also been developed. An excellent way of describing a product can be done by using flavour profiling (Meilgaard et al., 1991). Quantitative Descriptive Analysis provides a detailed description of all flavour characteristics in a qualitative and quantitative way. The method can also be used for texture. The panel members are handed a broad selection of reference samples and use the samples for creating a terminology that describes the product. Descriptive terms, such as paint, nutty, grassy, metallic are used for describing the oil on an intensity scale. A moderately oxidized fish oil is given fixed scores and used as a reference.

Profile of Fish Oil

Taste	Std
Fresh Fish	2
Amine	1
Oily	3
Sweet	2
Metallic	3
Grassy	3

Painty 2

Fruity 2

Remarks

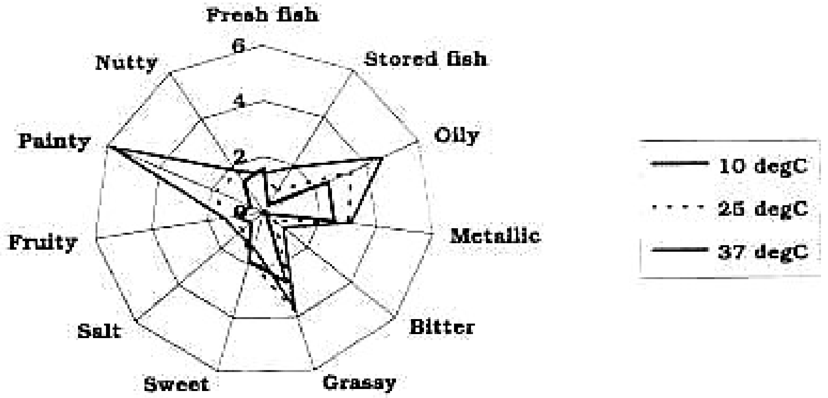
Taste as a whole (0 Unacceptable - 9 Neutral) 6

Profiling can be used for all kinds of fishery products, even for fresh fish when special attention is placed on a single attribute.

Statistics

Before conducting any experiment certain things to be planned beforehand, such as –

- Sensory analysis the experimental design (for example, number of panel members, number of samples, time aspects, hypotheses to test) and statistical principles.
- If not follow then experiments may lead to insufficient data and non-conclusive.
- For descriptive testing panel should consist 8-10 persons minimum.
- Test becomes more accurate when done in duplicate manner.
- Conducting a successful experiment adequate number of samples to be taken into consideration and can be done in random way.



Flavour profiles of a fish oil after 2 weeks of storage at various temperatures (Rorbaek *et al.*, 1993)

Training of Assessors

There are different ways to do sensory evaluation in all the methods.

- The more complexity assessment leads to increase in training difficulty varied from one degree to another.
- Triangle test needs small level of training.
- Sensory quality control is being performed by very less persons at the time of purchasing fish according to which they grade the fish.

- The person who is assessing must be trained in all the basic test and is aware of the common fish taste. Able to differentiate between off-flavour and taints.
- Basic skills can be trained in 2 days training program.

Biochemical Methods

This method deals with the evaluation of seafood quality with ability to create quantitative standards. It helps in eliminating the critics like products of marginal quality.

The following is an overview of some of the most useful procedures for the objective measurement of seafood quality. Woyewoda *et al.* (1986) have produced a comprehensive manual of procedures (including proximate composition of seafood).

Amines - Total Volatile Basic Amines

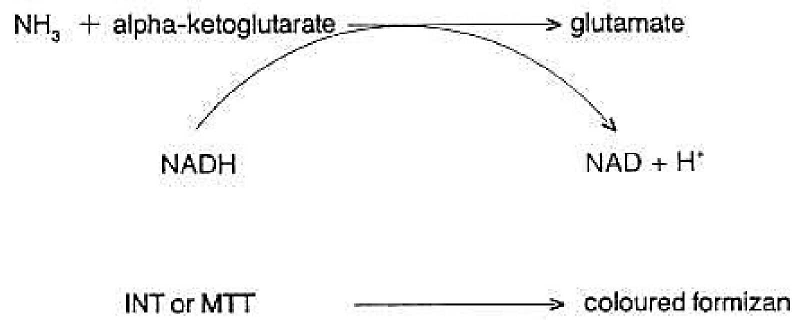
Total Volatile Basic Amines (TVB) is one of the most widely used measurements of seafood quality. It includes the measurement of trimethylamine (produced by spoilage bacteria), dimethylamine (produced by autolytic enzymes during frozen storage), ammonia (produced by the deamination of amino-acids and nucleotide catabolites) and other volatile basic nitrogenous compounds associated with seafood spoilage. Although TVB analyses are relatively simple to perform, they generally reflect only later stages of advanced spoilage and are generally considered unreliable for the measurement of spoilage during the first ten days of chilled storage of Cod as well as several other species. They are particularly useful for the measurement of quality in Cephalopods, such as Squid, Industrial Fish and Crustaceans.

Ammonia

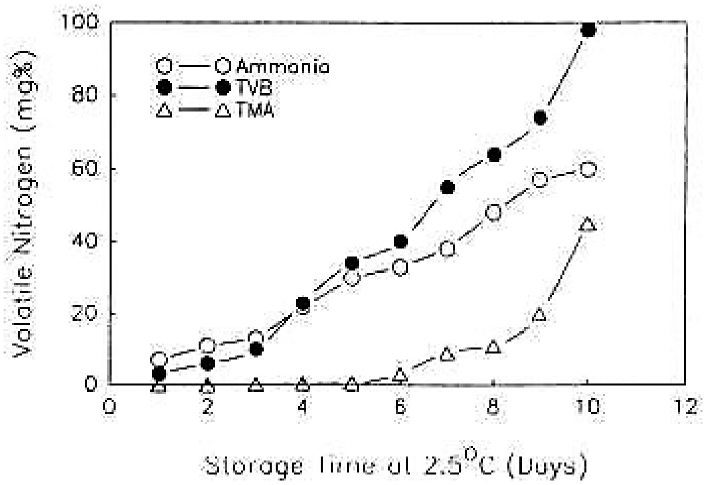
Ammonia is formed by the bacterial degradation/deamination of proteins, peptides and amino- acids. It is also produced in the autolytic breakdown of Adenosine Monophosphate (AMP) in chilled seafood products. Although ammonia has been identified as a volatile component in a variety of spoiling fish, few studies have actually reported the quantification of this compound since it was impossible to determine its relative contribution to the overall increase in total volatile bases.

Recently, two convenient methods specifically for identifying ammonia have been made available. The first involves the use of the enzyme glutamate dehydrogenase, NADH and Alpha-Ketoglutarate. The molar reduction of NH3 in a fish extract yields one mole of Glutamic Acid and NAD which can be monitored conveniently by absorbance measurements at 340 nm. Test kits for ammonia based on glutamate dehydrogenase are now available. A third type of ammonia test kit is available in the form of a test strip which changes colour when placed in contact with aqueous extracts containing ammonia (ammonium ion). LeBlanc and Gill (1984) used a modification of the glutamate dehydrogenase procedure to determine the ammonia levels semi-quantitatively without the use of a spectrophotometer,

but with a formazan dye, which changed colour according to the following reaction:



Where INT is iodontrotetrazolium and MTT is 3 - [4,5-dimethylthiazol-2-yl] 2,5 diphenyl tetrazolium bromide.



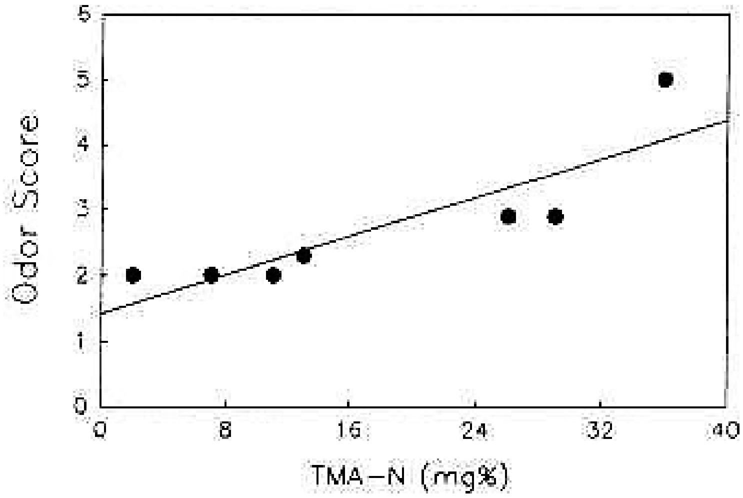
Effect of storage time on production of ammonia. TVB and TMA in short finned squid *(Illex illecebrosus),* adapted from Gill (1990)

Trimethylainine (TMA)

Trimethylamine is a pungent volatile amine often associated with the typical 'Fishy' odour of spoiling seafood. Its presence in spoiling fish is due to the bacterial reduction of Trimethylamine Oxide (TMAO) which is naturally present in the living tissue of many marine fish species. Although TMA is believed to be generated by the action of spoilage bacteria, the correlation with bacterial numbers is often not very good. This phenomenon is now thought to be due to the presence of small numbers of 'Specific Spoilage' bacteria which do not always represent a large proportion of the total bacterial flora, but which are capable of producing large amounts of spoilage-related compounds, such as TMA. One of these specific spoilage organisms, *Photobactetium phosphoreum,* generates approximately 10 - 100 fold the amount of TMA than that produced from the more commonly-known specific spoiler, *Shewanella putrefaciens*.

As mentioned above, TMA is not a particularly good indicator of edibility of herring quality but is useful as a rapid means of objectively measuring the eating

quality of many marine demersal fish. The correlations between TMA level or more preferably, TMA index (where TMA index = log (1 + TMA value)). Below figure illustrates the relationship between odour score and TMA level for Iced Cod. The linear coefficient of determination was statistically significant at the $P < 0.05$ level.



Relationship between odour score and TMA levels for iced cod. The straight line was fitted by linear regression analysis ($P< 0.05$) and all data points were averages of data obtained for three individual Cod, adapted from Wong and Gill (1987) .

Dimethylarnine (DMA)

The most common method for DMA analysis is a colorimetric determination of the DMA in deproteinized fish extracts. The Dyer and Mounsey (1945) procedure is still in use today although perhaps more useful is the colorimetric assay proposed by Castell *et al.* (1974) for the simultaneous determination of DMA and TMA, since both are often present in poor quality frozen fish. The chromatographic methods including gas-liquid chromatography (Lundstrom and Racicot, 1983) and high performance liquid chromatography (Gill and Thompson, 1984) are somewhat more specific, and are not as prone to interferences as the spectrophotometric methods. Gas chromatographic analysis of headspace volatiles has been proposed as a non- destructive alternative for amine determinations.

Biogenic Amines

Fish muscle has the ability to support the bacterial formation of a wide variety of amine compounds which result from the direct decarboxylation of amino-acids. Most spoilage bacteria possessing decarboxylase activity do so in response to acidic pH, presumably so that the organisms may raise the pH of the growth medium through the production of amines.

$$\text{Quality Index} = \frac{\text{ppm histamine} + \text{ppm putrescine} + \text{ppm cadaverine}}{1 + \text{pm spermidine} + \text{ppm spermine}}$$

They found that as the quality index ratio increased, the sensory scores on the canned product decreased.

Later, Farn and Sims (1987) followed the production of histamine, cadaverine and putrescine in skipjack and Yellow Fin Tuna at 20°C and found that cadaverine and histamine increased exponentially after an initial lag period of about 36 hours. However, putrescine increased slowly after an initial lag period of 48 hours. Levels of cadaverine and histamine increased to maximum levels of 5-6μg/g tuna but the authors reported that the absence of such amines in raw or cooked product did not necessarily mean that the products were not spoiled.

Nucleotide Catabolites

Several other approaches have been proposed for the analysis of individual or combination of nucleotide catabolites but none are more reliable than the HPLC approach. A word of caution is perhaps in order with regard to the quantitative analysis of nucleotide catabolites. Most methods proposed to date involve deproteinization of the fish samples by extraction with perchloric or trichloracetic acids. It is important that the acid extracts are neutralized with alkali (most often potassium hydroxide) as soon as possible after extraction to prevent nucleotide degradation in the extracts. Neutralized extracts appear to be quite stable even if held frozen for several weeks. One advantage of using perchloric acid is that the perchlorate ion is insoluble in the presence of potassium. Thus, neutralizing with KOH is a convenient method of sample 'Clean-Up' before HPLC analysis and this procedure helps to extend the life of the HPLC column. Also, it should be noted that nucleotide determination on canned fish does not necessarily reflect the levels in the raw material. Gill *et al.* (1987) found recoveries of 50%, 75%, 64% and 92% for AMP, IMP, INO and HX standards which were spiked into canned tuna prior to thermal processing.

Several unusual but innovative approaches utilizing enzymatic assays have been proposed over the years and are shown below in Table.

NOTES

Analyte(s)	Principle	Advantages	Disadvantages
HX	Enzymes (Xanthine Oxidase, X0) Immobilized on a Test Strip	Rapid Simple to use outside the lab	Semi -Quantitative Only capable of measuring HX (later stages of spoilage)
HX, INO	Test Strip, With Immobilized Enzymes	Rapid Simple to use outside the lab	Semi-Quantitative Poor Reproducibility Limited to HX and INO (later stages spoilage)
IMP, INO, HX	Enzyme-Coated Oxygen Electrode	Rapid Accurate	More complicated and time consuming than Test Strip Technology.
K-Index	Coupled Enzyme Assay "KV-101 Freshness Meter"	Rapid Results comparable to HPLC	Requires Enzymes and Reagents Cost Factor
K-Index	Enzyme-Coated Oxygen Electrode "Microfresh"	Rapid Results comparable to HPLC	Cost Factor

The factors which have been shown to affect the nucleotide breakdown pattern include species, temperature of storage and physical disruption of the tissue.

Microbiological Methods

This method deals with the bacteria or organisms present and to justify the hygienic factors in the fish which includes temperature abuse and handling procedure related to hygienic conditions. This does not give any data regarding the eating quality and freshness.

Earlier methods were laborious, time-consuming, and expensive, and require skill in execution and interpreting the results. Lots of microbiological methods have been developed out of which some of them can be used when large number of samples to be analysed.

Total Counts

This parameter is synonymous with Total Aerobic Count (TAC) and Standard Plate Count (SPC). The total count represents, if carried out by traditional methods, the total number of bacteria that are capable of forming visible colonies on a culture media at a given temperature. If a count is made after systematic sampling and a thorough knowledge of the handling of the fish before sampling, temperature conditions, packaging, etc., it may give a comparative measure of the overall degree of bacterial contamination and the hygiene applied. However, it should also be noted that there is no correlation between the total count and presence of any bacteria of public health significance. A summary of different methods used for fish and fish products is given below.

Common Plate Count Agars (PCA) are still the substrates most widely used for determination of total counts. However, when examining several types of seafood a more nutrient rich agar (Iron Agar, Lyngby, Oxoid) gives significantly higher counts than PCA (Gram, 1990). Furthermore, the iron agar yields also the number of hydrogen sulphide producing bacteria, which in some fish products are

the specific spoilage bacteria. Incubation temperature at and above 30°C are inappropriate when examining seafood products held at chill temperatures. Pour plating and a 3-4 day incubation at 25°C is relevant when examining products where psychrotrophs are the most important organisms, whereas products where the psychrophilic Photobacterium phosphoreum occurs should be examined by surface plating and incubation at maximum 15°C.

Microscopic examination of foods is a rapid way of estimating bacterial levels. By phase contrast microscopy the level of bacteria in a sample can be determined within one log-unit. One cell per field of vision equals approximately 5 -105cfu/ml at 1000 X magnification. The staining of cells with acridine orange and detection by fluorescence microscopy has earned widespread acceptance as the Direct Epifluorescence Filter Technique (DEFT). Whilst microscopic methods are very rapid, the low sensitivity must be considered their major drawback.

Bacterial numbers have been estimated in foods by measuring the amount of bacterial Adenosine Triphosphate (ATP) (Sharpe et al., 1970) or by measuring the amount of endotoxin (Gram-Negative Bacteria) by the Limulus Amoebocytes Lysate (LAL) test (Gram, 1992). The former is very rapid but difficulties exist in separating bacterial and somatic ATP.

Methods for Determination of the Content of Bacteria in Seafood

Method	Temperature, °C	Incubation	Sensitivity, cfu/g
Plate Count or Iron Agar	15-25	3-5 days	10
"Redigel"/"Petrifilm™ SM"	15-25	3-5 days	10
Microcolony-DEFT	15-30	3-4 hours	10^4-10^5
DEFT	-	30 min.	10^4-10^5
ATP	-	1 hour	10^4-10^5
Limulus Lysate Test	-	2-3 hours	10^3-10^4
Microcalorimetry/Dye Reduction Conductance/Capacitance	15-25	4-40 hours	10

Several methods (microcalorimetry, dye reduction, conductance and capacitance) used for rapid estimation of bacterial numbers are based on the withdrawal of a sample, incubation at high temperature (20-25°C) and detection of a given signal. In microcalorimetry the heat generated by the sample is compared to a sterile control, whereas in conductance and capacitance measurements of the change in electrical properties of the sample, as compared to a sterile control, is registered. The time taken before a significant change occurs in the measured parameter (heat, conductance, etc.) is called the Detection Time (DT). The DT is inversely related to the initial number of bacteria, i.e., early reaction indicates a high bacterial count in the sample. However, although the signal obtained is reversely proportional to the bacterial count done by agar methods, it is only a small fraction of the microflora that give rise to the signal and care must be taken in selection of incubation temperature and substrate.

Spoilage Bacteria

The total number of bacteria on fish rarely indicates sensory quality or expected storage characteristics. Different peptone-rich substrates containing ferric citrate have been used for detection of H2S-producing bacteria, such as ***Shewanella putrefaciens***, which can be seen as black colonies due to precipitation of FeS (Levin, 1968; Gram *et al*., 1987). Ambient spoilage is often caused by members of Vibrionaceae that also will form black colonies on an iron agar to which an organic sulphur source is added (e.g., Iron Agar, Lyngby). No selective or indicative medium exists for the ***Pseudomonas spp.*** that spoil some tropical and freshwater fish or for ***Photobacterium phosphoreum*** that spoil packed fresh fish. A conductance-based method for specific detection of ***P. phosphoreum*** is currently being developed. The presence or absence of pathogenic bacteria is often evaluated by methods based on immuno- or molecular biology techniques.

Spoilage Reactions

Several spoilage reactions can be used for evaluation of the bacteriological status of fish products. As described above, agars on which H2S producing organisms are counted have been developed. During spoilage of white lean fish, one of the major spoilage reactions is the bacteriological reduction of trimethylamine oxide to trimethylamine (Liston, 1980; Hobbs and Hodgkiss, 1982).When TMAO is reduced to TMA several physical changes occur: the redox-potential decreases, the pH increases and the electrical conductance increases. The measurement of such changes in a TMAO containing substrate inoculated with the sample can be used to evaluate the level of organisms with spoilage potential; thus the more rapid the change occurs the higher the level of spoilage organisms.

Detection time, has been found to be inversely proportional to the number of hydrogen sulphide producing bacteria in fresh aerobically-stored fish, and rapid estimation of their numbers can be given within 8-36 hours.

Pathogenic Bacteria

Several pathogenic bacteria may either be present in the environment or contaminate the fish during handling. A detailed description of these organisms, their importance, and detection methods is given by Huss (1994).

The bacteria most frequently described as fish pathogens are *Aeromonas*, *Edwardsiella*, *Pseudomonas*, *Shewanella*, *Mycobacterium*, *Streptococcus*, and *Flavobacterium*, of which some are common in Polish waters.

Microbial Inspection

The main cause of fish spoilage is the activity of microorganisms. To assess the freshness of various kinds of aquatic products, TVC (Total Viable Counts) is old and very helpful method.

On the basis of diverse storage conditions like temperature, time and atmosphere most of the countries have set their standards, guidelines and specifications for evaluating the freshness of fish.

An accurate method for detecting the fish freshness and remaining shell life of fish can also be predicted. Generally speaking, there are many microorganisms in fish flesh just after capturing and the initial value of TVC is usually approximately 102–104 CFU/g, the term CFU is abbreviation of Colony Forming Unit.

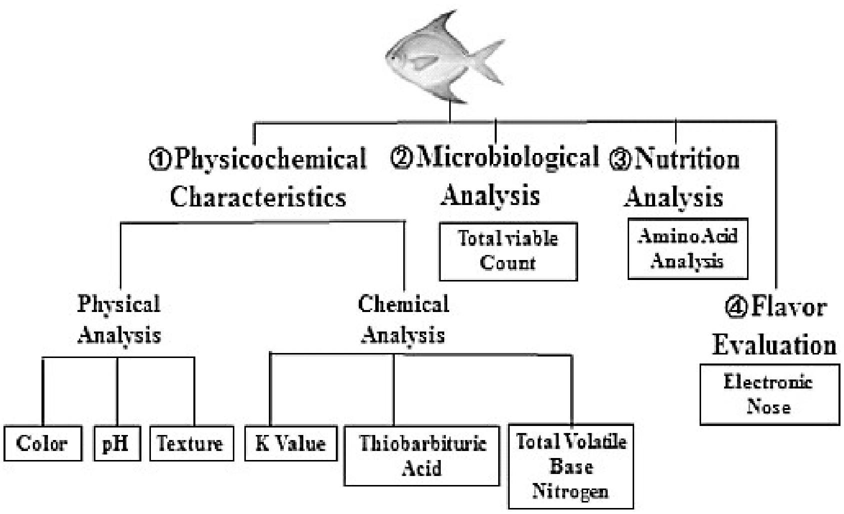
TVC values are used in various studies with the help of a process name Microbial deterioration which greatly contributes to the post-mortem of fish and remaining shell life.

O¨ zogul *et al.* (2005) studied the shelf life of Eel based on the TVC acceptability limit of 106 CFU/g and showed that the shelf life of Eel was about 13–14 days in ice and —six to seven days in boxes without ice, and the fish started to spoil after five days because of bacterial activity.

Thereafter, Chantarachoti *et al.* (2006) compared the total plate counts on fish of Alaska pink salmon stored at 14°C and stored in slush ice, and the results showed that aerobic bacteria counts for fish stored at 14°C increased from 3.4 $lgCFU/cm^2$ (zero day) to 4.8 $lgCFU/cm^2$ (three days) and for fish stored in slush ice the counts ranged from 3.4 $lgCFU/cm^2$ (zero day) to 5.5 $lgCFU/cm^2$ (16 days). In a recent study, Song *et al.* (2011) declared that the TVC values were 5.74 and 4.66 log CFU/g on the day of sensory rejection under two different storage conditions linked to chilled and partial freezing storage. TVC values were also used to correlate with sensory assessment.

Chemical Measurements

Chemical measurements mainly associated with the chemical composition changes of fish are a kind of important and indispensable method for determining and evaluating the freshness of fish. And chemical measurements normally refer to moisture measurement, volatile compounds measurements, protein changes, lipid oxidation, ATP decomposition, and K value measurement.



3. ESTIMATION OF PROTEIN, LIPID, CARBOHYDRATE AND SALT CONTENT IN FISH.

Estimation of Protein

Fish has been an important source of protein all around the world. Protein is calculated by measuring the volume of nitrogen instead of measuring protein directly due to the time consuming process.

As all the substances which have nitrogen do not include protein so whatever quantity is measured of nitrogen is termed as crude protein. Further to this true protein is free amino acids, tririmethylamine oxide and its decomposition products, and other substances.

About 16 per cent of nitrogen content is protein, so the nitrogen content in a sample fish is converted to cure protein by multiplying 6.25. But like dogfish have a high urea level, and urea has high nitrogen content so this calculation gives us wrong estimation of protein.

Factors to be considered for choosing a method -

1. **Sensitivity**- If protein is being detect at a very low level then methods needs to be described as sensitive one.
2. **Speciûcity**- The efficiency level of detecting protein
3. **Time**- Time taken or sent assay completion and interpretation of results.

Methods for Estimation of Protein

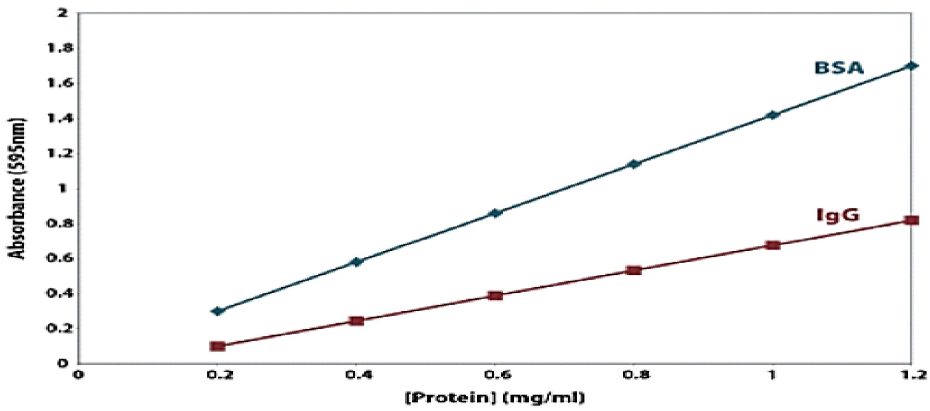
Biuret Method: Due to the need of high protein levels from 1-20 mg, the sensitivity of this method is very low. Copper sulfate, Sodium hydroxide, and Potassium Sodium Tartrate are the reagent used under this method. In this, protein reacts with alkaline copper complex and becomes violet in color. The protein can then be estimated by reading the absorption at 540nm. This method takes 20-30 minutes to complete. This method does not rely on amino acid composition and hence can measure all protein samples with accuracy. The main disadvantage of this method is that buffers with Tris, Ammonia interferes with the reaction.

BCA Assay: This method is highly sensitive and detects proteins at a low concentration of 1 µg. In this method, Copper ions bind to Nitrogen's in protein and the complex is then bound to bicinchoninic acid resulting in the change of color to purple depending on protein concentration. This method is sensitive to some chemicals. This method takes the longest time approximately an hour to get the end result. Common interfering substances are lipids, carbohydrates, Iron, impurities from glycerol, etc.

UV Absorption: Sensitivity of this method is moderate. It can detect proteins in the range of 50-100 µg. In this method, no reagents are required, the liquid protein sample is monitored under UV absorption at OD 280nm. Recently, simple machinery like Nanodrop and pico drop are used where even 1 µl of the protein sample is enough to determine protein concentration. This method takes less time, within 10 minutes.

Lowry Assay: Lowry assay is one of the old methods used for protein estimation developed by Oliver Lowry (1951). This is a highly sensitive and gives accurate results. It detects proteins at low concentrations of 2-5 μg. In this method, ûrst, the copper ions are reduced under alkaline conditions and forms a complex with peptide bonds of the protein. This complex then reduces Folin-Ciocalteau reagent and results in the change in color to deep blue and absorption can be measured at 650-750nm. It takes about 45-60 minutes for completion of the process.

Bradford Assay: This method was developed by Marion M Bradford (1976) and widely used. This is a very sensitive method and simple dye binding assay. This method uses Coomassie brilliant blue-250 dye that binds with negatively charged protein molecules. The dye color changes based on protein concentrations and the absorption is measured at 595nm. This method takes very less time compared to other regent based assays. Within 10-15 min the results can be obtained. Using standard curve protein concentration can be estimated in no time. The main disadvantage of this method is less speciûcity to interfering substances like SDS, Triton x-100, etc.



Features

- Sensitivity: Linear responses over the range of 0.5μg-50μg protein
- Flexible Protocols: Suitable for tube or titre plate assays
- Ready to use assay reagents and no preparation required
- Long shelf life, stable for 12 months

Kjeldahl Method

- Place 15 g of potassium sulphate and 0·5 g of copper (II) sulphate in an 800 ml Kjeldahl flask.
- Weigh accurately about 2 g of the prepared sample (1·5 g if rich in fat, 0·5 g if fish meal) on a filter paper and transfer both to the flask. Add carefully 25 ml of concentrated sulphuric acid and mix by swirling the flask.
- Place the flask in an inclined position on a suitable heating device in a fume cupboard. Heat carefully until foaming has ceased and the contents have

become liquefied. Digest by boiling gently, occasionally rotating the flask, until the liquid is completely clear and of a light blue colour; boil gently for a further 1½ hours.

- Total digestion time should be not less than 2 hours. Cool the contents of the flask to about 40°C and cautiously add 50 ml of distilled water. Mix and allow to cool.
- Transfer the contents of the flask to a 250 ml standard flask, rinsing several times, and make up to 250 ml. Transfer 50 ml to a suitable steam distillation apparatus.
- Put 25 ml of 4 per cent boric acid solution in a conical flask and place under the condenser so that the outlet dips into the liquid.
- Add 35 ml of 33 per cent sodium hydroxide solution to the distillation flask. Steam distil for 4 minutes after the first drop of distillate, or until the distillate is no longer alkaline.
- Lower the conical flask so that the condenser outlet is above the liquid level and distil for a further minute.
- Add to the flask 4 drops of indicator (0·2 g methyl red and 0·1 g methylene blue in 100 ml ethanol) and titrate to a grey colour with exactly 0·1 M hydrochloric acid.
- Repeat the analysis with a second 50 ml portion of digest.

Nitrogen content is calculated by:

$$N(\%) = \frac{0 \cdot 7(V_1 - V_0)}{M}$$

Where V1 is the mean volume in ml of 0·1 M hydrochloric acid required for fish.

V0 is the mean volume in ml of 0·1 M hydrochloric acid required for blank.

M is the weight in grams of the portion taken of the sample.

Nitrogen is finally converted to crude protein by multiplying by 6·25.

Estimation of Lipid Content

In fish lipid content ranges according to species, age, sex, season and location. Lipids are those components which are soluble in organic solvents but insoluble in water. It plays major role along with proteins as mode of metabolic energy for growth like reproduction, movement and migration.

According to OECD TG 305 following two methods are suitable for extraction and measurement of total lipids in fish.

- Bligh and Dyer Method
- Smedes Method

Bligh and Dyer Method

1. Firstly we weighed 5-10g wet sample in a pre-weighed 100 mL- conical flask.
2. Secondly, add 20 mL methanol (MeOH) and 10 mL chloroform (CHCl3).
3. Blend the sample for 2 min with an Ultra Turrax mixer.
4. 10 millilitres CHCl3 will be added a second time.
5. For 1 min shake the mixture vigorously.
6. 18 mL of distilled water will be added (including the water already in the sample).
7. Vortex the mixture again for 1 min.
8. Separate the two layers by centrifugation for 10 min at 450 g in a thermostatic centrifuge at 20°C.
9. The lower layer will be transferred to a pear-shaped flask with a Pasteur pipette.
10. A second extraction will be done with 20 mL 10% (v/v) MeOH in CHCl3 by vortexing for 2 min.
11. After centrifugation, the lower CHCl3 phase will be added to the first extract.
12. Evaporate the sample to dryness (with a rotavapor).
13. The residue will be further dried at 104°C for 1 hour.
14. Record the extracted weight and calculate the lipid content.

Below mentioned steps to be taken into consideration:

- Volume of solvents should be adjusted to the sample size.
- Wet fish samples should be homogenized with chloroform and methanol in a proportion not higher than: 1g tissue : 1 mL chloroform : 2 mL methanol.

Smedes Method (Based on de Boer *et al.* 1999)

1. The 5-10g wet sample will be weighed in a pre-weighed 100 mL-centrifugation glass beaker.
2. 16 mL of propan-2-ol, and then 20 mL of cyclohexane will be added to each centrifugation glass beaker using 20 mL pipettes.
3. The sample will be homogenized with the solvents for 2 minutes using Ultra Turrax.
4. x mL of deionized water will be added to obtain a total of 22 g water including the water already in the sample, x can be calculated according to the following equation:

$$\mathbf{x}\ [\text{mL}] = 22 - \frac{\text{sample wet weight [g]} * \text{water content [\%]}}{100}$$

5. The sample will again be homogenized for 1 minute using Ultra Turrax.
6. When an emulsion occurs, this will be dissolved by adding of HCl (37%) drop wise from a Pasteur pipette while gently stirring the mixture with a glass rod.
7. The phases will be separated by centrifuging the mixture for 5 ± 1 minutes at 440 - 460 g.
8. The upper cyclohexane phase will be separated and volumetrically quantified, and transferred to a 10 mL glass centrifugation beaker.
9. After addition of 20 mL of a mixture of cyclohexane/propan-2-ol (87:13, w/w) to the remaining lower watery phase, the phases will again be homogenized for 1 minute and afterwards separated by centrifuging the mixture for 5 ± 1 minutes at 440 - 460 g.
10. Again, the upper cyclohexane phase will be separated, volumetrically quantified, and transferred to the same 40 mL glass centrifugation beaker holding the first extract of the corresponding sample.
11. The combined cyclohexane extracts will be concentrated by gentle nitrogen stream to approx. 3 mL; the concentrated extract will be quantitatively transferred into a pre-weighed 10 mL wide mouth glass flask.
12. The glass centrifugation beaker used for concentrating the extract will be rinsed with 3 mL of the cyclohexane/propan-2-ol mixture using ultrasonication, and the solvent will be added to the corresponding wide mouth flask.
13. The solvents in the flask will be evaporated to dryness (with a rotavapor).
14. The flask will be further dried for 1 hour at 105.
15. After cooling to room temperature in a desiccator, the dried flask will be weighed, and the lipid content calculated according to:

$$\frac{(\text{Weight of Dry Flask [g]} - \text{Weight of Empty Flask [g]}) * 100}{\text{Wet Weight}} = \frac{\text{Lipid Content in \% of}}{\text{Wet Weight}}$$

Volume of solvents should be adjusted to the sample size. Wet fish samples should be homogenized with propan-2-ol and cyclohexane in a proportion not higher than: 1g tissue:1.6 mL propan-2-ol:2 mL cyclohexane.

Carbohydrate

Fish muscle normally contains only traces of carbohydrates, in the form of sugars, sugar phosphates and glycogen. Some other tissues, such as liver contain larger amounts as glycogen, and most molluscan shellfish also contain a fair amount of glycogen. There is no single method suitable for determining total carbohydrate in all tissues and, apart from the indirect infrared method mentioned earlier under protein, the methods are not straightforward. For these reasons it is common to estimate Carbohydrate (C) by difference.

$$C\ (\%) = 100 - P - W - F - A$$

Where P is percentage protein (nitrogen × 6-25)

W is percentage water

F is percentage fat

A is percentage ash

Because of possible accumulation of errors in the four separate analyses, the accuracy of such an estimate may be low, especially if the amount is small.

Estimation of Salt Content in Fish

Salt is being as most commonly used as an antimicrobial agent in all the fish products.

Raw Materials

Commercial samples of dry fish varieties, such as ***Stolephorus spp., Scomberomorus spp., Leiognathidae spp., Sillago spp., Decapterus russelli*** and ***Lates calcarifer***.

Estimation of NaCl %

Modified Mohr's method was followed for determination of NaCl %. 1 G of sample was powdered and dissolved in 30 mL of boiling Milli-Q water. 1 mL of 5% potassium chromate solution was added as indicator and titrated against 0.1 N silver nitrate solution. The initially yellow solution turned into white precipitate on formation of silver chloride. The end point occurred when all chloride ions precipitated and excess silver ions reacted with chromate ions of the indicator to form red-brown precipitate of silver chromate. The titre value was noted and substituted in the formula mentioned below to express NaCl content in percentage,

$$\text{NaCl \%} = \frac{\text{Titer value} \times \text{Normality of } AgNO_3 \times 58.4 \times 100}{\text{Weight of sample} \times 1000}$$

Estimation of Sodium (mG/ G)

Sodium content was determined by mathematical calculation from the NaCl content (Moncada, 2013) using the below mentioned formula,

$$\text{Sodium}\left(\frac{mG}{G}\right) = \frac{\text{NaCl \%}}{100} \times \text{weight of sample used (G)} \times \text{sodium \% in NaCl} \times 1000$$

Table 1 *Sodium Chloride (%), Sodium (mG/100G) and Moisture (%) of Different Varieties of Commercial Dry Fish Samples*

Sample No.	Dry fish samples	NaCl (%)	Sodium (mG/ 100G)	Moisture content (%)
S1	*Stolephorus spp.*	18.44 ± 0.03^{g}	3687.67 ± 5.04^{g}	10.25 ± 0.03^{e}
S2	*Stolephorus spp.*	8.94 ± 0.04^{c}	1787.33 ± 8.35^{c}	14.38 ± 0.04^{f}
S3	*Stolephorus spp.*	2.93 ± 0.03^{a}	586.67 ± 5.93^{a}	3.83 ± 0.02^{a}
S4	*Leiognathidae spp.*	18.69 ± 0.01^{h}	3737.20 ± 1.74^{h}	18.58 ± 0.01^{h}
S5	*Leiognathidae spp.*	6.47 ± 0.05^{b}	1294.93 ± 9.54^{b}	22.47 ± 0.02^{i}
S6	*Sillago spp.*	15.72 ± 0.03^{f}	3143.20 ± 5.33^{f}	23.46 ± 0.02^{j}
S7	*Sillago spp.*	22.13 ± 0.08^{i}	4426.80 ± 5.56^{i}	7.19 ± 0.02^{c}
S8	*Sillago spp.*	26.82 ± 0.02^{k}	5363.60 ± 4.64^{k}	17.83 ± 0.02^{g}
S9	Scomberomorus spp	23.41 ± 0.03^{j}	4681.93 ± 5.28^{j}	5.71 ± 0.03^{b}
S10	Scomberomorus spp	18.66 ± 0.03^{h}	3731.00 ± 5.63^{h}	8.74 ± 0.03^{d}
S11	*Decapterus russelli*	12.83 ± 0.02^{d}	2565.80 ± 4.00^{d}	29.74 ± 0.03^{k}
S12	*Lates calcarifer*	13.47 ± 0.03^{e}	2694.13 ± 6.95^{e}	33.75 ± 0.01^{l}

The data are expressed as Mean ± S.E (n=3). The values bearing different superscripts in a column represent significantly different mean values ($P<0.05$)

In general dry fish is a heavily salted product due to traditional practices and need of salt for preservation. Doyle *et al.* (2010) stated that although NaCl performs important technological functions during the production of many meat, fish, dairy, and bakery products, some of these foods probably contain more salt than is necessary for high-quality characteristics. Out of the 12 commercial samples analyzed, 7 samples (S1, S4, S6, S7, S8, S9 and S10) were found to have NaCl concentration above 15%.

4. DETERMINATION OF STOCKING DENSITY AND FEED ASSESSMENT.

Fish occupy three dimensional spaces which is related to volume and measured in cubic metres. To determine stocking density is an important parameter in aquaculture operations. This directly related to the growth, survival and production of fishes. There is an inverse relationship between the growth of fishes and stocking densities as growth rate of fishes increases as the other one decreases and vice versa.

There is one term which is known as critical standing crop, it can defined as without hampering the fish growth maximum biomass a system can support. Following are the stocking methods which can be used for the maintenance of fish biomass.

1. Sequential Rearing
2. Stock Splitting
3. Multiple Rearing Units

Carriage Capacity – A pond / tank containing maximum weight of fishes with adequate space. This method is being adopted in fish farming. This is water management technique in which recirculation of water takes place through which maximum number of fishes can be accommodated like per square meter in pond and per cubic meter in tank.

According to the research, a static pond system has maximum stocking capacity of 1.8 kg of fish per meter square.For example, 2000 meter square of static pond water can carry 3600 kg of catfish.

Below is the calculation of a mono-culture system of catfish farming and to maintain water quality, each fish should ne fed above 20 g.

Suppose static pond volume is 2000 meter square and we intend to produce average size of 900 grams of catfish at the end of culturing season with average mortality rate of 5 percent.

2000 meter square of water volume multiplied by 1.8 divided by 900 gram plus 5 percent expected mortality rate i.e. (2000m2 x 1.8kg/900g) + 5% (2000m2 × 1.8kg/900g)

(3600/0.9) + 5%

4000+ 5% of 4000

4000+ 200 = 4200 juvenile

Fish Stocking Density in Aquaponic Systems (Critical Standing Crop)

Below are the specifications for above mentioned system.

- **System Type:** (DWC) Deep Water Culture
- **Filtration:** Biofilter and Clarifie
- **Oxygen Level:** High (>9ppm)
- **Optimum Stock Density:** 21-30 Kg/1000 Liter
- **Number of Fish:** 72 per 1000 Litre

With the Hybrid Filtration:

- **System Type:** (DWC) Deep Water Culture
- **Filtration:** Hybrid
- **Oxygen Level:** High (>9ppm)
- **Optimum Stock Density:** 31-40 Kg/1000 Litre
- **Number of Fish:** 100 per 1000 Litre

This is the recommended starting fish stock level for a DWC (Deep Water Culture) system with biofilter and clarifier filtration system. As the biofilters become more effective (usually over 6 months) the stock level can be increased.

- **System Type:** (DWC) Deep Water Culture
- **Filtration:** Clarifier (Biofilter take 3 to 6 months to become fully effective)
- **Oxygen Level:** Medium to High (>8-9 ppm)
- **Optimum Stock Density:** 11-20 Kg/1000 Litre
- **Number of Fish:** 43 per 1000 Litre

Feed Assessment

Feed assessment means the moment each and every feed ingredient entered into the mill, verification of quality standard being set takes place along with the close supervision on the ingredients quality through the storage period before usage and during its on-going processing.

The quality control process continues it goes into storage as compound feed.

Quality Control Procedures

Raw Materials

This ensures that minimum contract requirements are being met for raw materials. It gives us knowledge regarding the exact composition of raw materials and also the required nutritive value present in the level of toxic substances. This is done with the help of the nutritionist, management personnel and the quality control manager. They assess the specifications pertaining to nutrient quality, cost and the quality desired in the feedstuffs.

Most contemporary feeds for livestock are formulated with the aid of a computer which calculates the cheapest product obtainable from available raw materials within constraints laid down for the nutritive value, toxicity, and palatability of the final product. Such least cost formulations are possible only when the composition of each raw material is known with a high degree of precision and when good quality control is maintained.

- **Preliminary Inspection**

All the materials at the mill should be under go through physical inspection by the following:-

(a) Evidence of Wetting - Mould Growth Confirms Water Damage.

(b) Presence of Scrap Metals, Stones, Dirt, or Other Non-Biological Contaminants.

(c) Presence of Insects.

The content of moisture available in feed grains should be tested with one of the procedures commercially acknowledged. If any of them exceeds more than 13 % then the chances of insect prone increased and the same should be separated from other stored ingredients. One important thing to remember is that materials with high moisture should be taken into the store only after drying.

- **Sampling**

In this sampling of bagged ingredients is done with a spear probe. From one corner of the bag to another, probe is inserted diagonally as well as horizontally in a best way out. In lots of 1-10 bags, all bags are sampled. In larger lots, 10 percent of all bags are sampled. Materials received in bulk are sampled by using a scoop, according to the size of the consignment. For smaller than 10 t consignments, two samples per ton are taken. Larger consignments, up to 100 t, require one sample per ton or one sample for every two tons depending on the size of the consignment.

Samples taken in the above manner should be pooled, thoroughly mixed, and then reduced in size by quartering to between 1 and 2 kg in weight.

Oil cakes and other coarse materials are sampled by random selection of pieces from different parts of the entire consignment. Five pieces per ton of materials are considered sufficient. The pieces should then be ground, mixed thoroughly, and the sample reduced in size to between 1 and 2 kg - as mentioned above.

Samples submitted to the quality control laboratory should be placed in tightly sealed containers. Prior to chemical analysis, the samples must be reduced to a powder using a Waring type blender or a mortar.

- **Tests Required**

For feedstuffs likely to contribute to both the protein and energy content of the final product, a “Weende” proximate analysis is usually conducted. This determines

the moisture, crude protein, lipid, crude fibre, ash, and nitrogen-free extract content of the feedstuff.

Additional tests should be carried out on materials having a high ash content to determine the proportion of acid insoluble ash present, thus providing an indication of the amount of sand or other dirt present. This will also enable detection of any deliberate contamination of expensive feedstuffs, such as fishmeal. Salt (NaCl) analysis of fish meal is also required for these same reasons, as well as to avoid excessive levels of sodium in the final diet.

Calcium and phosphorus determinations are routinely conducted on mineral feeds, such as bone meal, calcium phosphate and calcium carbonate sources. Other feedstuffs such as fish meal, and meat and bone meal are also analysed for these two mineral elements.

- **Other Tests**

The protein requirements of some cultured animal species are more exacting than others. For example, fish not only require higher protein levels in their diets but the protein quality requirements are also higher. The determination of amino acid content is a lengthy and complicated process requiring highly specialized, and expensive, equipment. However, it is advisable to test feedstuffs, such as fish meal, for lysine availability.

Certain feedstuffs contain natural toxins that, at high enough levels, are growth inhibitory and sometimes fatal to the animal consuming them. Principal among these are:

(a) Urease

An enzyme found in raw soybeans which produces toxicity through the hydrolysis of urea to toxic ammonia. The toasting process in soybean meal manufacture destroys the enzyme.

(b) Gossypol

An endogenous toxin present in the gland of cottonseed which persists during production of the meal unless removed by a special process, or, unless the cottonseed is a glandless variety.

(c) Isothiocyanates

Cyanogenic Glycosides are found in linseed and cassava. Much of the toxicity is eliminated during processing of the raw material.

(d) Aflatoxin

Aflatoxin is a class of extremely potent toxins produced by the mould Aspergillus flavus. Aflatoxin may be present in any materials produced and stored under hot and humid conditions and is usually found in groundnut cake, palm kernel cake, copra cake, and maize (which have not been properly dried after harvest).

Molasses is a common feedstuff in the tropics. Periodic analysis is necessary to establish its sugar content. Molasses sometimes contain an undesirably high level of potassium and occasional checks on this should be conducted.

Trace mineral and vitamin supplements form an important part of balanced diets for both fish and livestock. The availability of these feed components from reliable sources makes it quite unnecessary to perform routine checks on their quality unless large volume purchases are involved. Sending samples to a reliable commercial laboratory obviates the great expenses of equipping a laboratory for such testing.

Finished Products

This process involves determining the manufacturing process to ensure that ingredients were added in the proportions required by the formulation. Inhomogeneity of the final product, due to improper mixing or unwanted ingredient separation, thus, can be detected.

- **Preliminary Inspection**

Most modern mills are equipped with sieves and magnets along the material flow lines for removal of tramp metal, rocks, and other scrap contaminants. However, smaller operations may not have such features, and physical inspection of the finished product should be carried out to determine the presence of such contaminants. Any detection of foreign contaminants should be brought to the attention of the mill supervisor who could then determine if the contaminants originated in the raw material or if they were the result of improper maintenance within the mill premises.

- **Sampling**

To detect product inhomogeneity and significant ingredient separation during the manufacturing process, sampling should be obtained during bagging-off time by taking a handful from every fifth bag of 40-50 bags and pooling the individual samplings. Testing for variability is best conducted by probing the bottom, middle, and top of a bag with a short probe. Tests may be made on each sample, or the samples from the same bags may be combined and mixed before analysis depending upon the question to be answered.

The size of finished feed samples need only be half that required for raw materials, i.e., 0.5-1.0 kg after quartering. Samples submitted for chemical analysis should be placed in tightly sealed containers.

- **Tests Required**

It is not uncommon for feed to be despatched to the customer on the same day it is manufactured, a day or two before chemical analysis on the product is completed. Therefore, one senses the importance of proper raw material quality control and the apparent irrelevance of quality testing on finished products.

Nonetheless, quality control of finished products is necessary because it serves two important functions:

(a) It checks the manufacturing process,

(b) It checks the quality specifications, or claims, established for the finished products.

If raw material quality control is properly conducted and if process control is adequate, then the only chemical tests required of finished products on a regular basis are for moisture and crude protein. Periodic inspections on other components of the "Weende" proximate analysis should also be scheduled.

RAW MATERIAL AND FINISHED PRODUCT STORAGE

Both raw materials and finished feeds undergo deteriorative changes during storage. For raw materials, these changes represent a direct economic loss because of the resultant decrease in nutritive value. Finished products undergo changes which not only lower their nutritive value below minimum specifications but also affect their palatability and appearance.

Due to seasonal fluctuations in availability and price, it is often necessary to maintain large inventories of raw materials if demands are to be continuously satisfied at stable price levels. Six-month inventories of stocks of imported raw materials are not uncommon..

METHODS OF ANALYSIS

(a) Moisture

Weigh and place 4-5 g of the sample in a covered, flat, aluminium dish. Dry to constant weight at 100-105°C in a drying oven.

$$\text{Moisture content (\%)} = \frac{\text{Weight fresh sample - Weight dry sample}}{\text{Weight fresh sample}} \times 100$$

(b) Crude Protein (Kjeldahl Method)

Reagents

- Sulphuric acid (98%), nitrogen free,
- Potassium sulphate, reagent grade,
- Mercuric oxide, reagent grade,
- Paraffin wax,
- Sodium hydroxide, 40% solution,
- Sodium sulphide, 4% solution,
- Pumice chips,
- Boric acid/indicator solution.
- Add 5 ml of indicator solution (0.1% methyl red and 0.2% bromocresol green in alcohol) to 1 litre saturated boric acid solution, and
- Hydrochloric acid standard solution (0.1N).

Apparatus

(a) Macro Kjeldahl Digestion and Distillation Units.

(b) Kjeldahl Flasks (500 ml capacity or larger).

(c) Conical Flasks, 250 ml.

Method

Accurately weigh 1 g of sample into a digestion- flask. Add 10 g potassium sulphate, 0.7 g mercuric oxide (pre-measured catalyst tablets containing these two reagents are available), and 20 ml sulphuric acid. Heat the flask gently at an inclined angle until frothing subsides and then boil until the solution clears. Continue boiling for an additional half hour. If the frothing is excessive, a small amount of paraffin wax may be added.

On cooling, add about 90 ml distilled water, recool, add 25 ml sulphide solution, and mix. Add a small piece of boiling chip to prevent bumping and 80 ml of sodium hydroxide solution while tilting the flask so that two layers are formed. Connect rapidly to the condenser unit, heat, and collect distilled ammonia in 50 ml boric acid/indicator solution. Collect 50 ml of distillate. On completion of distillation, remove the receiver (wash condenser tip) and titrate against standard acid solution.

Calculation

Nitrogen content of sample (%)

$$= \frac{(\text{ml acid} \times \text{normally of standard acid})}{\text{wt of sample(g)}} \times 0.014 \times 100$$

(c) Crude Fat

Reagents and Equipment

- Petroleum Ether (b.p. 40-60°C).
- Extraction Thimbles.
- Soxhlet Extraction Apparatus.

Method

Weigh into an extraction thimble 2-3 g of the dried sample (residue from dry matter determination can be used). Place the thimble inside the Soxhlet apparatus. Connect a dry pre-weighed solvent flask beneath the apparatus and add the required quantity of solvent and connect to condenser. Adjust the heating rate to give a condensation rate of 2 to 3 drops/s and extract for 16 h. (The extraction time may be reduced to a minimum of six h by increasing the condensation rate.) On completion, remove the thimble and reclaim ether using the apparatus. Complete the removal of ether on a boiling bath and dry flask at 105°C for 30 min. Cool in a desiccator and weigh.

Calculation

Crude fat (% of DM)

$$= \frac{\text{weight of fat}}{\text{weight of sample}} \times \frac{100}{1}$$

(d) Free Fatty Acids

Reagents and Apparatus

- Ethyl Alcohol
- Phenolphthalein (1% Solution in Alcohol).
- Sodium Hydroxide (0.25N).
- Stoppered Flasks, 250 ml.

Method

Weigh oil or fat into a stoppered flask and add 50 ml alcohol previously neutralised by adding sufficient 0.25N sodium hydroxide to give a faint pinkish colour with phenolphthalein (2 ml). Titrate with sodium hydroxide and vigorous shaking until a permanent faint pink colour appears.

Calculation

Free Fatty Acids % (as Oleic Acid)

$$= \frac{\text{g oil or fat}}{7.05} \times \text{volume of 0.25N NaOH used in titration}$$

Retain extracted sample for crude fibre analysis and extracted fat for free fatty acid determinations.

(e) Crude Fibre

Reagents

- Sulphuric Acid Solution (0.25 N).
- Sodium Hydroxide Solution (0.313N).
- Antifoam Reagent (Octyl Alcohol).
- Ethyl Alcohol.
- Hydrochloric Acid, 1% v/v.

Apparatus

- Beakers, 600 ml Tall-Sided
- Round-Bottom Flask Condenser Unit.
- Buchner Flasks, 1 litre.
- Buchner Funnels. Hartley 3 Section Pattern, Crucibles, Silica with Porous Base, and Rubber Cones.

Method

Weigh about 2 g of the dried, fat-free sample into a 600 ml beaker. Add 200 ml of hot sulphuric acid, place the beaker under the condenser, and bring to, boiling within 1 min. Boil gently for exactly 30 min, using distilled water to maintain volume and to wash down particles adhering to the sides. Use antifoam if necessary. Filter through Whatman No. 541 paper in a Buchner funnel, using suction, and wash well with boiling water. Transfer residue back to beaker and add 200 ml hot sodium hydroxide solution. Replace under the condenser and again bring to boil within 1 min. After boiling for exactly 30 min, filter through porous crucible and wash with boiling water, 1% hydrochloric acid and then again with boiling water. Wash twice with alcohol, dry overnight at 100°C, cool, and weigh. Ash at 500°C for 3 h, cool, and weigh. Calculate the weight of fibre by difference.

Calculation

Crude Fibre (% of Fat-Free DM)

$$= \frac{\text{(weight crucible + dried residue)-(weight crucible + ashed residue)}}{\text{(weight of sample)}} \times 100$$

Ash

Weigh a 2 g sample into a dry, tared porcelain dish and then place in a muffle furnace at 600°C for 6 h. Cool in a desiccator and weigh.

Calculation

Ash (%)

$$= \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

Acid Soluble and Insoluble Ash

Reagents and Apparatus

(a) Hydrochloric Acid (1-2.5 v/v).

(b) Filter Paper, Ashless.

(c) Dishes, Porcelain.

Method

Use the residue obtained from the ash determination. Boil with 25 ml hydrochloric acid, taking care to avoid spattering, filter through ashless filter paper, and wash with hot water until acid free. Place filter paper and residue into a dry, tared porcelain dish and place in a muffle furnace at 600°C for 2 h or until carbon free.

Calculation

Acid insoluble ash (%)

$$= \frac{\text{weight of acid - treated ash}}{\text{weight of sample}} \times 100$$

Thioglucoside Determination

The method described will give approximate thioglucoside content but does not allow the individual thioglucosides and isothiocyanates to be determined.

Reagents and Apparatus

(a) Barium Chloride (5% Solution).

(b) Volumetric Flasks, 600 ml.

(c) Steam Bath.

Method

To 10 g meal (de-fatted by Soxhlet extraction) add 250 ml distilled water, hydrolyse at 54°C for 1 h and then boil for 2h, keeping volume constant. Filter, retaining filtrate, and wash residue three times with 50 ml hot water. Add washings to initial filtrate and make up volume to 600 ml. Precipitate barium sulphate by heating and adding excess barium chloride solution. Leave on a steam bath for a few hours and then filter. Ash in a muffle furnace and then weigh precipitate.

Calculate approximate thioglucoside content as:

$$\% \text{ trioglucoside} = \frac{(\text{M. wt. trioglucoside})(\text{Wt. of BasO}_4)}{(\text{M. wt. BasO}_4)(\text{Sample Wt.})} \times 100$$

Aflatoxin Analysis

A method of aflatoxin analysis is outlined below which is suitable for materials, such as groundnut meal, coconut meal, and palm kernel meal.

Reagents

(a) Chloroform (Reagent Grade).

(b) Diethyl Ether (Reagent Grade).

(c) Chloroform/Methanol Mixture (95/5 v/v),

(d) "Celite", Diatomaceous Earth,

(e) Kieselgel 'G' (Merck),

(f) Qualitative Standard

Helps to distinguish aflatoxin spots from other fluorescent spots which may be present.

Apparatus

(a) Thin Layer Chromatography Plates, 20 × 20 cm.

(b) UV Lamp, Peak Emission at 365.

(c) Bottles, Wide-Mouthed, 250 ml.

(d) Micropipettes.

(e) Shaking Device.

Method

Weigh 10 ml of material into a wide mouthed bottle and thoroughly mix in 10 ml of water. (If high fat material is used, a prior Soxhlet extraction with petroleum ether will be necessary.) Add 100 ml of chloroform, stopper with a chloroform resistant bung, and shake for 30 min. Filter the extract through "Celite", take 20 ml of filtrate and make up to 25 ml (solution a). Take another 20 ml of filtrate and concentrate to 5 ml (solution b).

Prepare thin layer plates by shaking Kieselgel 'G' (100 g) with water (220 ml) for 20 min and apply the mixture to the plates with a suitable apparatus to a depth of 509m. Leave for 1 h, then dry at 100°C. Spot 10 and 20m of solution b, and 5 and 10 m of solution a onto a plate, together with a qualitative standard spot, in a line 2 cm from the bottom of the plate and at least 2 cm in from each side. Carry out the spot application in subdued light.

Develop the plate in diethyl ether to a height of 12 cm. Allow to dry in subdued light then redevelop the plate in chloroform methanol (95/5, v/v) to a height of 10 cm from the baseline. Examine the plate in a dark room, 30 cm from the UV source. The presence of a blue fluorescent spot at Rf 0.5 to 0.55 indicates aflatoxin B (check that the standard spot also lies in this range). The presence of a second spot at Rf 0.45 to 5 indicates aflatoxin G. The toxicity level of a sample can then be classified in terms of aflatoxins B and G according to Table given below

Toxicity Levels for Alatoxins B and G

Volume Applied (m l)	Concentration of Aflatoxins (m g/kg)		Toxicity Level of Fluorescence Observed
	No Fluorescence	With Fluorescence	
5m (soln. a)	< 1000	> 1000	very high
10m (soln. a)	< 500	500-1000	High
10m (soln. b)	< 100	100-500	Medium
20m (soln. b)	< 50	50-100	Low

5. METHOD OF TRANSPOR1TATION OF FISH SEEDS

In aquaculture, transportation of fry, hatchlings and fingerlings of culturable species is a common necessity. Breeders of large adults transported very frequently for facilitating seed production.

At some of the places in the world, transport of fish seed in earthen pots taken as head loads or on slings from their collection centres to specialized market and to nurseries for stocking to serve as an ancient practice.

Certain developments have been made in transport technology considering following factors:

- While transporting process internal physiological mechanism of the fish and also make to sure the survival of fish to maximum extent.
- Study of the environmental parameters of the medium in which fish are transported.

It is the study of autecology of the fish related ambient conditions like ambient oxygen, pH, ammonia and physiological conditions like conditioning fish fry, starvation before transportation.

Causes of Mortality in Transport

Several factors responsible for mortality of fish in transport are as follows:

1. Depletion of dissolved oxygen in ambient water due to the respiration of fish and also due to oxidation of any organic matter (BOD load), including excreted waste of the fish, by micro-organisms.
2. Accumulation of free carbon dioxide (CO_2), resulting from respiration, and ammonia (NH_3) as excretory end product.
3. Sudden fluctuations in temperature.
4. Hyperactivity and stress due to handling and 'confined space' - these result in lactate accumulation and affect again lessening of blood oxygen capacity and also 'fatigue collapse'
5. Ion-osmotic imbalance due to stress.
6. Physical injury due to handling before transport and during transport.
7. Diseases.

Physiological Effects of Handling and Transport on Fish

Due to the stress in which live transport of fish seed and brooders are being catched and transferred with or without net from the nursery/farm into the equilibration system and further to transport container sometimes may lead to injury and immediate death.

In order to avoid the transport stress the physiological effects including hyperactivity should be taken care of as severity of stress would depend on the transport duration and chemical characteristics pertaining to the containers.

Increase in physical activity can cause immediate depletion of the energy enhancing the production of lactic acid. The lactic acid causes 'Acidocis' and consequent lowering of pH, which results in the liberation of CO_2. The acidic pH and increased pCO_2 in blood would cause reduced loading of oxygen in the blood (Bohr and Root effects), which would seriously impair the energy yielding mechanism of the fish and can cause collapse of the fish. Acidosis of the blood would affect the other bio-chemical reactions as well and can also cause fatigue collapse.

The surviving fish quite often would accumulate an oxygen debt, which under the crowded condition of the transport carrier, coupled with low oxygen conditions can again lead to the collapse and death of the fish. Therefore it is imperative that the fish caught for transport should be given sufficient time in high oxygenated water under optimal conditions for Conditioning of fish before transportation.

Conditioning of Fish before Transportation

Spawn, fry and older fish for long transportation have to be prepared or conditioned. The fish seed and broodfish are kept starving usually in a cloth 'Hapa' or other containers in a quiet corner of the fish pond or in relatively quiet water in a canal or river for a period of time before transferring them to the transport carrier.

The advantages of conditioning fish are:

(i) The fish become used to confined condition.

(ii) The fish are less excited and thus restrained in expenditure of energy.

(iii) The fish recover from the handling effect of capture - increased blood lactate level and decreased blood pH become normal - excited high metabolic rates (O_2 consumption, CO_2 production, N-excretion) become normal.

(iv) The fish recover from minor injury - mucus loss etc - Ion-osmotic balance upset by handling becomes normal.

(v) Gut evacuation takes place and during the period of transport the medium is not further contaminated by faecal matter.

(vi) Since the fish are starved there is exhaustion of available glycogen and this considerably reduces the chance for accumulation of high amount of lactic acid in blood during transport and consequent acidocis and collapse..

According to Saha and Chaudhury (1956), the depth of water where a conditioning enclosure is to be installed should be 30 to 35 cm. The period of conditioning depends on the size and health of the spawn (hatchling), fry and fingerlings. Jagannathan (1947) stated that fry need 48 to 72 hours of conditioning. Saha and Chaudhury (1956) agree with this observation and suggest that the period of conditioning can be applied to fingerlings in general.

Alikunhi (1957) remarked that about 6-hours conditioning is required for fry, whereas Srivatsava and Karamchandani, (1964) suggest that fry (8 – 23 mm) require 24-hours conditioning for transport in a limited volume of water (2 ml per fry). The fry were seen to survive even when oxygen concentration was 0.88 ppm during transport. It appears that in general a conditioning period of 6 – 24 hours would suffice for all species of fish - longer conditioning period should be given specially in cases where stress due to capture and subsequent handling is high. A point often ignored is that there is considerable ion-osmotic stress on all fish due to handling and in freshwater fish considerable amount of salt from the body is likely to be lost due to capture and handling. Addition of a little amount of NaCl in the ambient medium especially in the case of tilapias, might be of advantage, but the system has to be sufficiently modified since conditioning is often done in open waters.

Various types of conditioning containers are used, namely boxes made of wire meshes, bamboo or cane wicker work; barrels or boats with perforated

bottoms; temperary enclosures made of netting or bamboo matting, cloth 'Hapa', etc., any of these materials available conveniently may be used.

The temperature of the conditioning water should not be high - it should be preferably on the lower side of the optimal thermal range for the species. During conditioning and transportation fry and fingerlings should not be handled with bare hands - the slime over the fish body should remain intact. The loss of slime and scales would render the fish disadvantageous in maintaining osmotic balance and also fish are likely to get infected.

Methods of Packing and Transport

Transport carriers are of two types: (a) Open System comprising open carriers, with or without artificial aeration/oxygenation/water circulation and (b) Closed System having sealed air tight carriers with oxygen.

1. Open System

This system comprising open carriers, with or without artificial oxygen/water circulation. Earlier transport carrier is the earthen vessel known as traditional "Hundi" which is now interchanged by aluminium vessels. The most important benefit of using earthen hundies is that they keep the temperature of the water cool inside through evaporative cooling. Two types of earthen vessels used in Bengal – the smaller one of 20 cm diameter and 23 litres capacity carried as a head load or on a bamboo sling; and the larger are of 23 cm diameter and 32 litre capacity, used for transport by railways.

About 50,000 carp spawn are released in the small vessel and 75,000 in the larger (Saha and Chaudhury, 1956). In traditional transport in India, about 60 g of finely pulverised red soil is sprinkled over the water surface in the transport vessel and the vessels are shaken periodically during transport. The addition of red soil coagulates the suspended organic matter and keeps down the zone and extent of pollution (Basu, 1951). During transport, the bottom sediments are periodically removed by mopping them up with a rough cloth rope - the water is also partially renewed depending on the need. The addition of red soil and change of water permit transport of fry upto a duration of 30 hours. Besides pulverised earth other absorbent substances such as activated charcoal and 'Amberlit' resin can be also used as these absorb carbon dioxide, ammonia and other substances from the medium (Saha *et al.,* 1955).

The improved metal containers are better than the earthen carriers only because they are not breakable. The metal containers used are round vessels with a wide mouth, which can be closed with perforated pressed-in lids, the larger type being 53 cm in diameter at the base, 20 cm at the mouth and 38 cm high. To prevent denting and perhaps more to effect insulation, wooden covers are used on the metal containers; often the vessel is crated and kept wet during the journey.

Larger containers mounted on motor vehicles have also been in use. In some of these a semi-rotatory pump has been added producing sprays of water

over the water surface in the tank, through a delivering tube with two rows of holes at 45° to each other. Fish fry have been transported in such motor vans (semi-insulated) over a distance of 500 km with mortality less than 5%. Several other adaptations of open transport carriers mounted on motor vehicles are also in vogue.

In spite of being cheaper, open packing system for transport of fish seed is going out of fashion mainly because it involves continuous vigilance and frequent renewal of water during long journeys. It is obviously not worthwhile or economical to transport bigger fingerlings and adults in small packing units. For this purpose, truck-mounted open tanks with mechanical aeration and water circulation (as the one explained) have been in use successfully (Hora & Pillay, 1962; Patro, 1968; Berka, 1986). Galvanised iron drums of 180 L capacity, and open canvas containers (1 m × 1 m × 1.25 m) have been used successfully in transporting breeders. The galvanized iron drums are provided with an opening of 48 cm × 30 cm along the main body of the container for introduction of fingerlings and larger fish. The drums is often lined inside with foam rubber to avoid injury to the buffeting fish during transport. Recently plastic pools (250 litres and other sizes) have also been used for transporting fish.

In China and South-East Asia efficient open containers have been developed, some of traditional design, for the transport of fingerling and adult fish. In Indonesia water-tight tar-coated, plaited bamboo baskets capable of accommodating 10,000 fry of 5 cm in size, are used.

2. Closed System

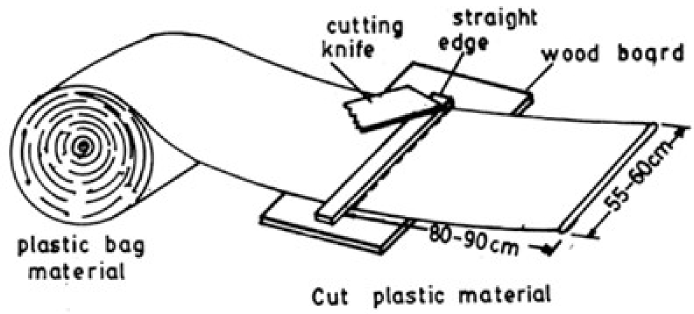
In this system the water surface is exposed to compressed air or pure oxygen introduced to fill the zone over the water surface in the carriers which are sealed air-tight can be better explained through given below figures.

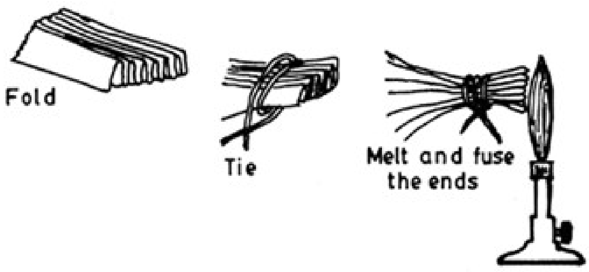
3. Sealed metal containers, rubber and plastic bags have been used for the purpose. In a metal container of galvanized iron (45 × 35 × 35 cm) with two airtight openings, one to let in oxygen and other to let out water, 100 – 200 fingerlings of 7 – 10 cm, or 30 – 40 fingerlings of 13 – 20cm length, can be transported for a journey of 12 hours (Khan, 1946). Eighteen litre tins with airtight screw-capped lids for filling and provided with tubes for drawing in oxygen and letting out water, have been used in CIFRI, Barrackpore, for transporting 1000 fry of 1 – 2 cm length during a 20-hour journey.

4. Polythene bags of various dimensions (74 cm × 46 cm or 65 × 45 cm - thickness 0.0625 mm) are now widely used in fish fry and fingerling transport. In this method the bag is first put into a tin or any rigid box of 18 – 20 litre capacity and the bag is filled upto S! of its capacity (6–7 litres) with water and the required number of seed is put into it and the bag is inflated with oxygen in high pressure from a cylinder, upto T! of the bag. The upper 10 – 15 cm of the bag is twisted, bent and tied securely airtight with a string. Carp hatchlings numbering 20,000 – 40,000,

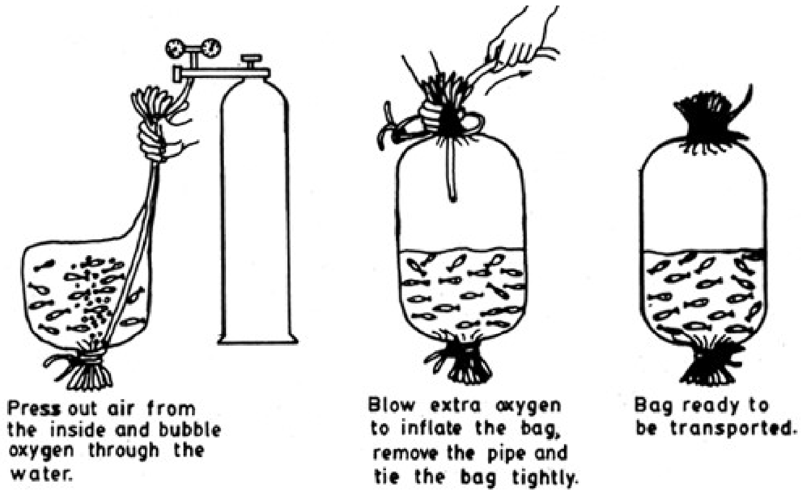
300 – 600 fry (30 – 40 mm) and 40 – 70 fingerlings per bag depending on the distance are packed and transported in this manner

5. For transport of larger fish (large fingerlings, brooders) large closed-system carrier tanks have been designed. Water being splashless in the tank is of special advantage, as explained, as the fish would not injure themselves much. Fry (1951) designed a smaller galvanized splashless carrier - the design here is simple and mainly consisted in the use of a rubber tubing (cycle tyre) which can be inflated on top to fill the space and also addition of ice cubes on top of the carrier to control temperature.



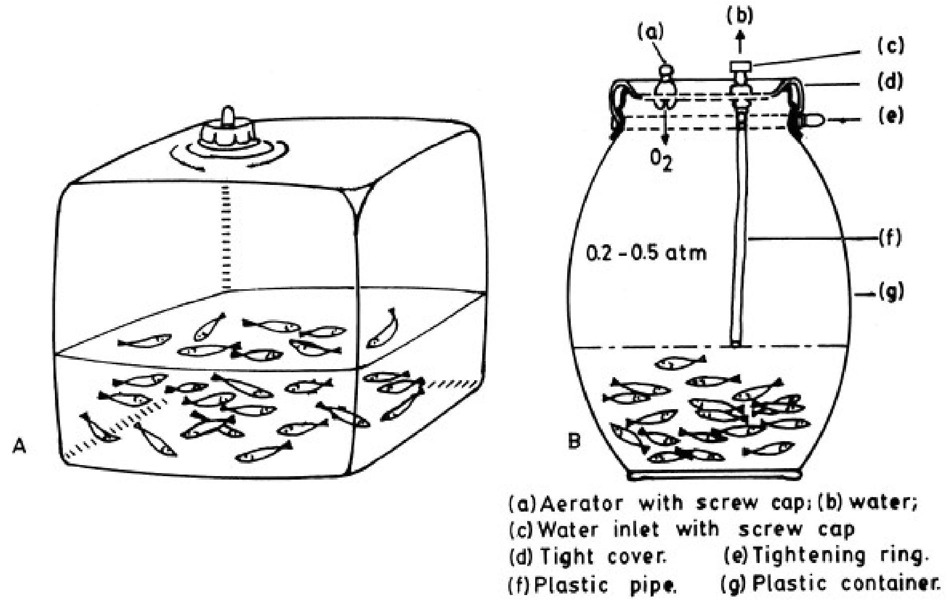


Procedure of closing the bottom end of a polyethylene sleeve (After Woynarowich and Horvath, 1980)



Procedure of filling the bag with water, stocking with the fish, displacing the air, introducing oxygen and closing the upper end. (After Woynarowich and Horvath, 1980)

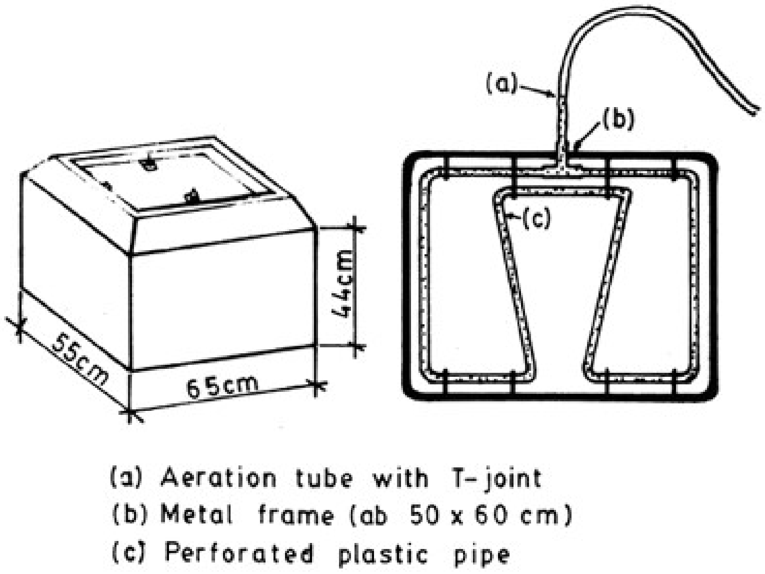
NOTES



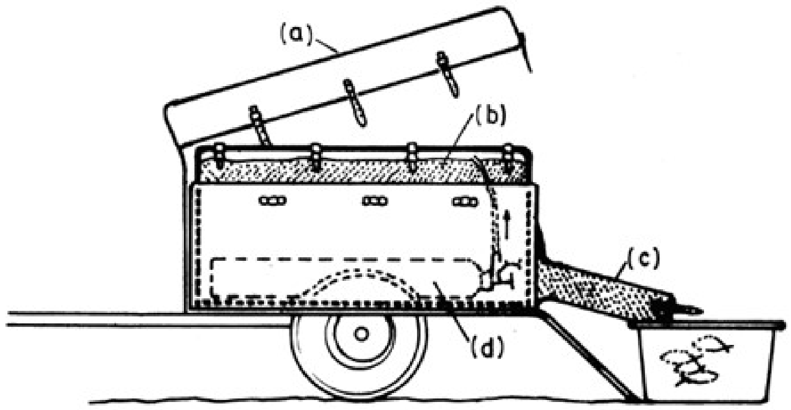
Sealed Plastic Containers

A. Container Volume 25 litres, the Oxygen-Inlet Valve is built in the screw cap.

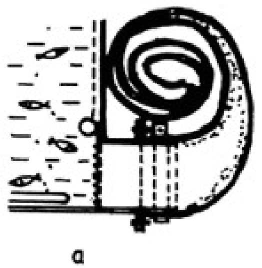
B. Container Volume 50–150 litres, Vertical Plastic pipe keeps water at the required level.



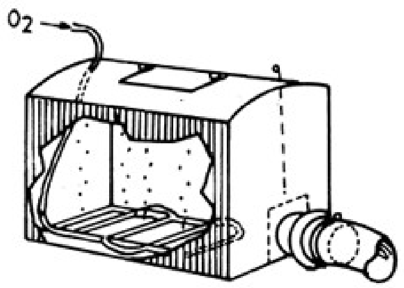
Small tank to be carried in a car. Container volume 100 – 150 litres; the aeration grate is adjusted to s fit the dimensions of the tank.



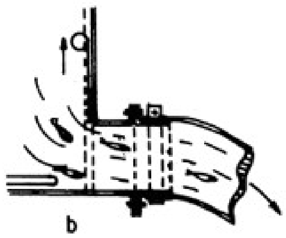
Passenger-car trailer for fish transport.



(a) During transport

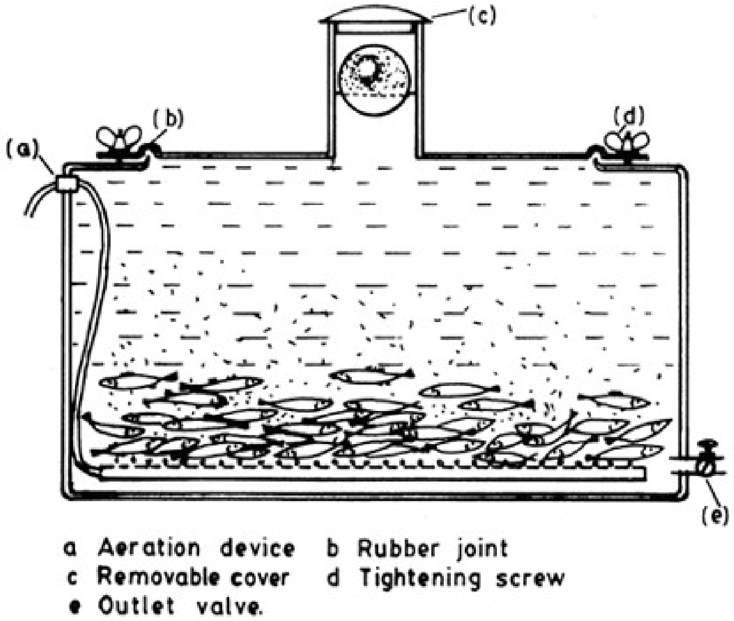


Transport tank with hopper

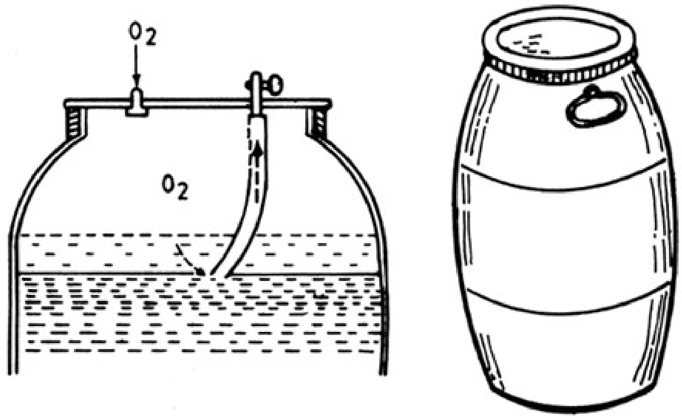


(b) Unloading

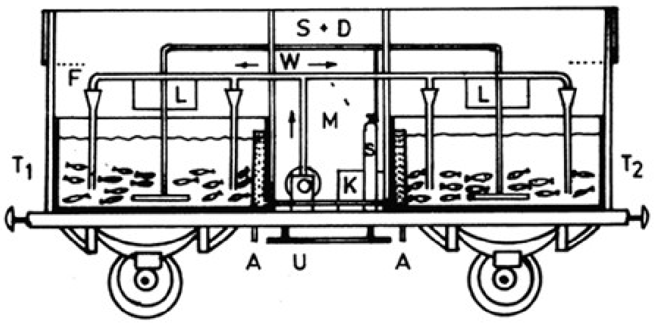
Transport tank with hopper.



Specialized tank for pike-perch transport.



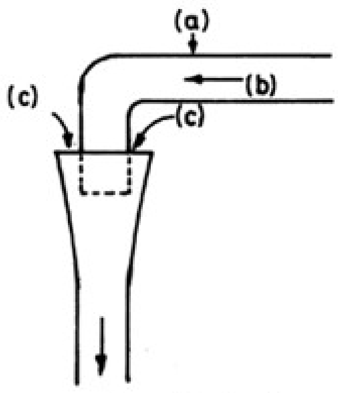
Plastic tank for pike-perch transport.



A fish transport tank wagon (From Berka, 1986)

NOTES

M - technical space and attendant's booth, U - pump and air compressor, T - transport tanks 1 and 2, S - oxygen cylinders, A - tank drain, F - water aeration, L - loading space, W - circulating water distribution system, S + D - oxygen or compressed-air distribution system.



A larger model is the splashless thank of Mammen (1967) which is a modified petrol tank of 1150 litre capacity with an auto-clave type of lid. It has a built-in aeration system for supplying compressed air which works on a belt driven by the engine of the transporting vehicle.

An oxygen cylinder is also carried as a stand-by for emergency. A total weight of 250 kg of fish can be transported at a time, fish weighing 60 kg and 90,000 fingerlings have been transported successfully in this splashless tank. The load ratio comes to about 1 kg of fish per 4.5 litree of water i.e. 1:4.5 (Mammen, 1967; Jhingran, 1975).

Patro (1968, see Jhingran, 1975) designed a live fish carrier, which comprises of an outer cylindrical chamber of 120 cm diameter open from top and a lightly smaller inner cylinder closed from top. The top of the inner cylinder is provided with an air vent and an oxygen valve. The outer chamber serves as a storage tank and is initially filled with water and fish to be transported let in.

The inner chamber which is slipped in from the upper open end of the outer chamber, serves as an oxygen holding chamber at its top and is also lined with foam rubber. This 'double barrel' carrier can transport 100 kg of live fish at a time. Once filled, the oxygen supply of the carrier lasts upto 5 hours (and indeed on the rate of utilization by fish) and has a unique advantage that a constant pO_2 is maintained throughout, which is determined by the weight of the inner cylinder. The whole unit is a modification of the laboratory gas supply unit, and is a good example that transports carriers can be made out of common material available and ingenuity of the users.

Use of Chemicals in Live Fish Transport

Drugs and chemicals are either used as tranquilizers and sedatives or as antiseptics and antibiotics. Sedatives are generally used for:

(i) Reducing metabolic rates, mainly oxygen consumption and excretion of carbon dioxide and ammonia.

(ii) Reducing excitability of fish and injury.

(iii) Convenience in handling fish.

The sedatives and drugs have to be used very carefully, for slight increase in dosage and/or exposure time can cause irretrievable loss of fish.

Use of Antiseptics and Antibiotics

Commonly used chemicals and their doses are indicated below

Methylene Blue	-	2 ppm
Acriflavin	-	10 ppm
Chloromycetin	-	8–10 ppm
Copper Sulphate	-	0.5 ppm
Sodium Chloride	-	3%
Potassium Permanganate	-	3 ppm

A prophylactic bath of fry and fingerlings in the above mentioned chemicals is recommended while handling the fish prior to transport, for prevention and spread of diseases - pathogens and parasites.

6. CRAFTS AND GEARS USED FOR FISHING

Usage of crafts and gears enhanced the production commercial bases. Successful rate of fishing largely depends on to how and which types of nets are used to capture the fish.

There are two main types of devices used to capture fishes in both marine and inland fisheries:

Nets or Gear — These are instruments used for catching fish.

Crafts or Boats — It provides platform for fishing operations, carrying the crew and fishing gears.

There are various types of gears and crafts used in different parts depending upon the nature of water bodies, the age of fish and their species. Some nets are used without craft, however, others are used with the help of crafts.

Crafts and Boats

There are many types of fishing crafts being successfully made and used for marine and inland fisheries.

A. Marine Fishing Crafts

Different crafts are used due to different conditions of sea on the east and west coasts.

I. Crafts used on the East Coasts

Catamaran

The word catamaran is originated from a Tamil word Kattumaram which means 'Lashing Timber'. It is used mainly on the east costs of Orissa from Kanyakumari. It is also used on northeast cost of Kerala. It is the most primitive, traditional, economical and efficient craft.

It is made by tying many wood logs in such a way that it takes the shape of a canoe, which consists of two main logs and two side logs cut into boat shape and held together with rope. The logs are held in position by loose rubber called Teppa. Generally the Catamaran is 5-10 metre long, 0.5 metre wide and 0.3 metre deep.

Types of Catamarans

i. Orissa and Ganjam Type

It is made by five logs pegged with wood. The logs are cut in boat shape and are not tied with rope.

ii. Coromandal Type

It is used in Tamil Nadu to capture flying fish of Nagapattanam. It is made by 3-5 logs. A modified type of Coromandal type is called Kolamaram, which is made by 7 logs.

iii. Andhra Type

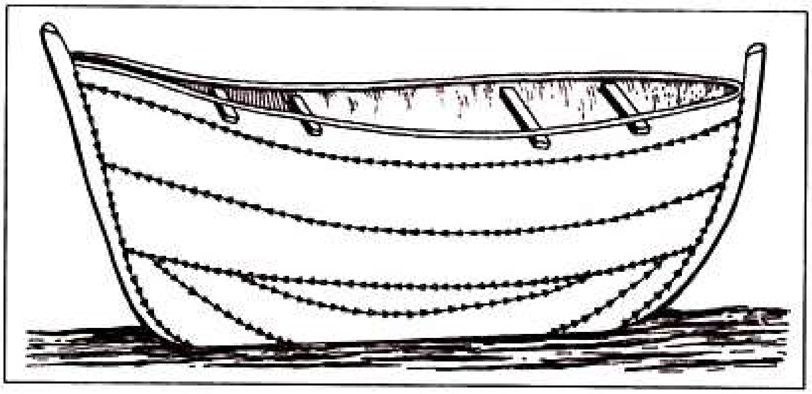
It is the modified form of Orissa type, larger in size—about 5-7 metres long, hence made by nine heavy side wood logs that are fitted with a median logs.

iv. The Boat-Catamaran

It is made of three wood logs tied in boat shape. It is used in Mandapam and Mukkun coastal regions.

(2) Masula Boat

It is a weakly constructed boat of about 8-12 metres long shown in figure below. It is used in clear weather near the shore. The Masula boat is keel less and frameless made by mango planks, which are stitched with palm leaf fibres. There are several variations of the Musula boat. In Orissa it is called 'Bar Boat' and in Andhra called Padava or Padgam.

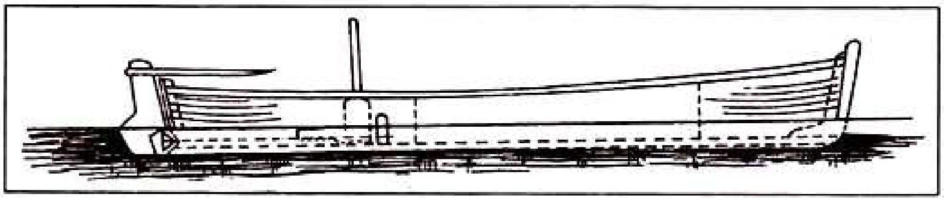


(3) Nauka and Dinghi

These boats are with carvings and are operated in West Bengal and Orissa. These are well-designed large boats measuring about 11-13 m x 2-3 m x 2 m in size.

(4) Tuticorin Boats or Fishing Luggers

These are operated in inshore waters and are used as cargo boats. They are carved boats of 11 m x 2m x 1m in size.

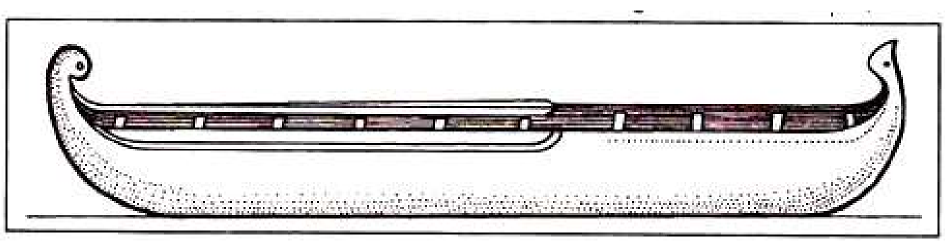


II. Crafts used on West Coasts

(1) Dugout Canoes

These are constructed from large wood logs. These logs are hollowed by scooping inner part. Their bottom is thicker than sides. They are operated on Kerala and Konkan coasts. The small boats of 5-10 m long are called 'Thonies', which are used for gill nets or drift fishing and for seining.

The large boat of 10-22 m long is called Vanchi or Odam and is used for operating variety of nets on Malabar Coast. Dug-out canoes are also operated on the west coast from Colachal to Kathiawar.



(2) Plank-Built Canoes

It is a kind of dug-out canoes. It is extended with planks on sides. They are popularly used on coast of Kerala, Karnataka and North Bombay.

(3) Outrigger Canoes

These are large size canoes of about 15 m long. It has narrow keel and single outrigger and extended out with planks. They are commonly called as Rampani, because they are used for the casting of the Rampani net for catching mackerels. They are operated on the Kanara and Konkan coasts. Small size outriggers are used between Bhatkal and Majali.

(4) Built-Up Boats

It is highly specialized indigenous fishing craft. They are commonly used along the Bombay coast and north Ratnagiri.

There are small variation according to different places, such as

(A) **Ratnagiri Type Boat:** This has pointed bow, straight and narrow keel and low gunwale.

(b) **Machwa:** It is provided with broad hull, straight keel and pointed bow. It is popular in Bassein hence called Bassien type.

(c) **Satpati or Galbati Type:** It has straight keel, high gunwale, medium pointed bow and broad beam. Satpati can be mechanized with a motor engine without any modification in design.

(d) **Broach Type:** It has flat bottom and is widely used in inshore and estuarine water.

(5) Coracle

It is used in rivers, reservoirs and canals for fishing. One or two fishermen can operate this craft. It is made like a round basin and its frame is made with split bamboos. The outer surface is covered with leather.

(6) Shoe Dhonie

It is shaped like a shoe. It is used both marine and inland fishery. It is constructed by teak wood with planks grooved with ribs and frames fitted with nails. It is used for fishing with gill nets.

(7) Kakinada Nava

It is commonly used for inshore fishing. It is made by teak wood. It is keel less but ribs are fitted in frame with nails. It is about 9-10 m in length.

Marine Fishing Gears

Various types of gears are used for fishing in sea. They may be of different size, shape and designs. These gears may be made by fishermen. They are also manufactured in cottage industries. The most commonly and widely used fishing gear are different types of nets.

They are used for catching large fishes offshore. The main type of nets being used are boat seine, shore seine, bag nets, fixed or stationary nets, drag nets, drift nets and cast nets.

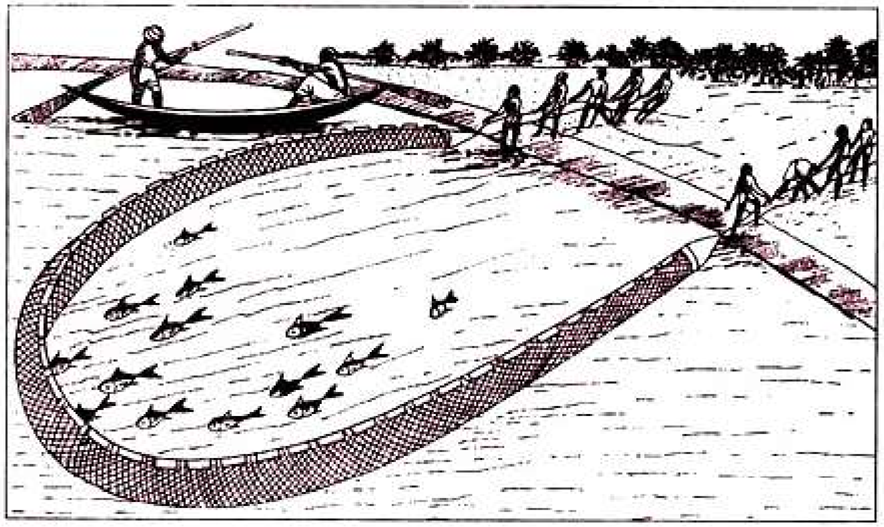
(i) Seines

These are specially designed and large fishing nets. They are generally used in running water. When they are spread in sea; they collect large numbers of fishes. Seines are rectangular in shape mounted on wire. They are spread vertically in the water. Seines are of two types, boat and shore seines.

(a) **Boat Seine:** These nets are conical in shape provided with wings. The mesh of the nets is smaller in center and increase in size towards outer ends of flanks. This seine is operated in sea by catamarans or boats. The seine traps the fishes. Towing is done with the help of coir.

(b) **Shore Seine:** It is operated from seashore. It is popularly called as Ber Jal in Orissa, Pedda or Alivi vala in Andhra coast, Periya vala or Mada valai in coromandel coast and Kara valai in the Gulf of Mannar. It is a conical bag-like with two wings.

One end of net is kept fixed to shore and other end is spread into sea with the help of a boat, in the form of semi-circular fashion. When the net is filled up with fishes, the two ends are slowly dragged by group of fisherman.



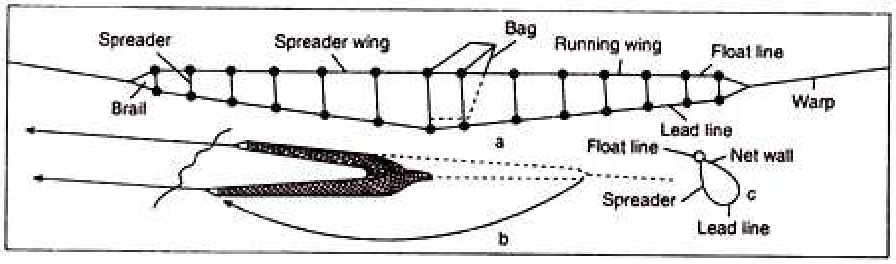
(ii) Danish Seine

It is also called drag seine. It is used in deep waters and do not reach to surface. It has small wings.

(iii) Beach Seine

It is also called haul seine. It has two wings made by strong twines. Both wings are joined to a central bag. The ends of wings are tapering and conne-cted to the wraps either directly or by means of spreader (brail) of strong pole. The mesh size is smaller in the bag than in the wing. Beach seine has side, i.e., float line and lead line. The float line contains appropriate floats whereas lead lines carry sinkers.

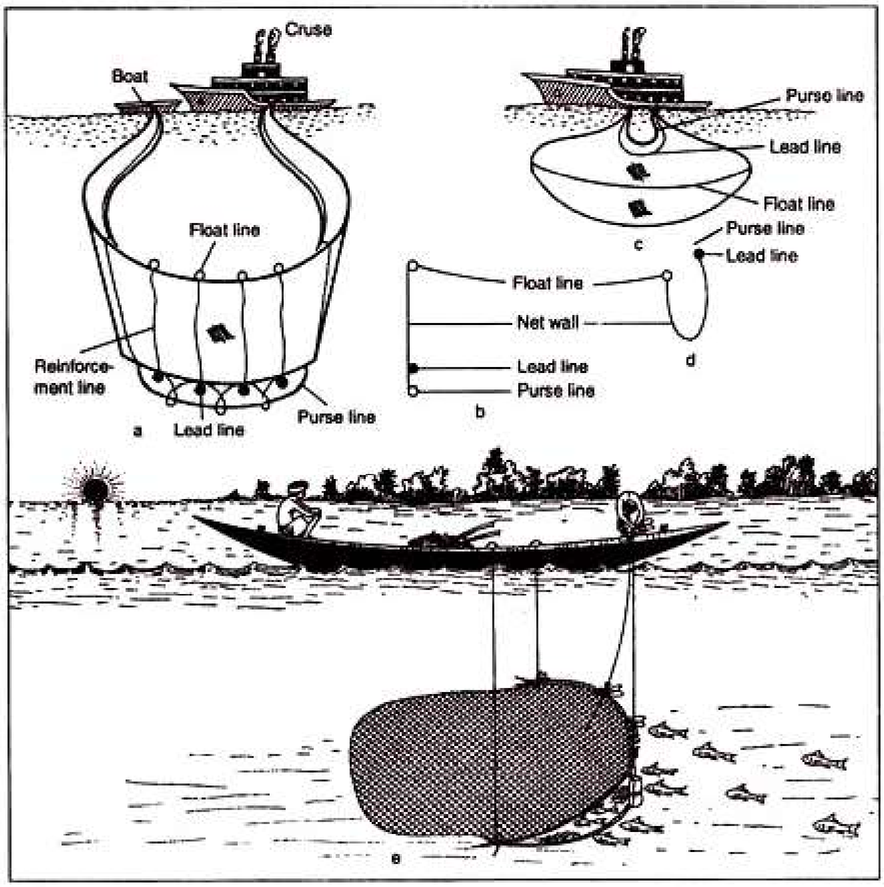
The net is used in such a way that its one wing remains on the beach. The other wing is spread on right angle in such a way that, when it is dragged slowly it encircles that part of water section. Both float and lead line do not allow fish to escape.



(iv) Purse Seine

It is used to catch pelagic and migratory fishes. It is purse-like. It has two main lines—float line, which remains on the surface and a lead line, which sinks into the water, but does not touch the bottom. The fishes are trapped and do not escape because net is pursed during the operation.

At the time of use, one end of the net is tucked with a boat and another end is laid down with the help of a cruise making a circle and bringing this end back to the boat. The net then takes the shape of purse.



(v) Trap Nets

They are generally used for fishing in shallow waters. Trap nets are strong and made in various shapes and sizes. These nets may be stationary or fixed. Its lower part is cylindrical while upper part is conical. Interior region of the net contains one or two cone-shaped necks to prevent escape of fish. Large trap nets are called a pound net, which has a chamber with a wide gate.

(vi) Drop Net

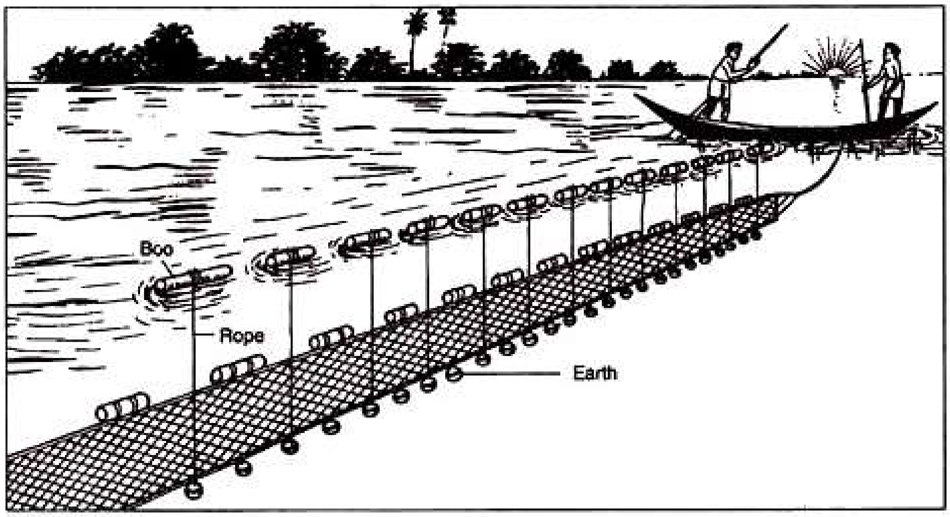
It is square in shape and mounted with to supple loops at the corners that tied in a cross at the top and is attached to a pole. Drop net is operated with a boat. It is dropped and pulled to catch fishes.

(vii) Cast Net

It is a circular and cone shaped net. It is spread from the edges of water. Its circumference is attached to lead line while its centre is attached with a rope. The net assumes shape of umbrella when it is spread on the water. When the net sinks to the bottom it is pulled and fishes are collected.

(viii) Drift Nets and Gill Nets

These types of nets are made by nylon materials. Gill nets are kept overnight in the water and then dragged. The fishes get entangled in the meshes. There are two types of nets—simple and trammel nets.



(ix) Simple Gill Net

These are loosely woven nets. When spread in water, fishes get entangled in mesh. If the fishes try to escape the twine of the net get mingled in the gills of fishes. The fish is said to be gilled (captured by gills) and hence the name given ‘Gill Net’.

(x) Trammel Gill Net

It has a float line at the top and a dead line at the bottom; two walls are attached to these lines. It is generally operated to catch small fishes.

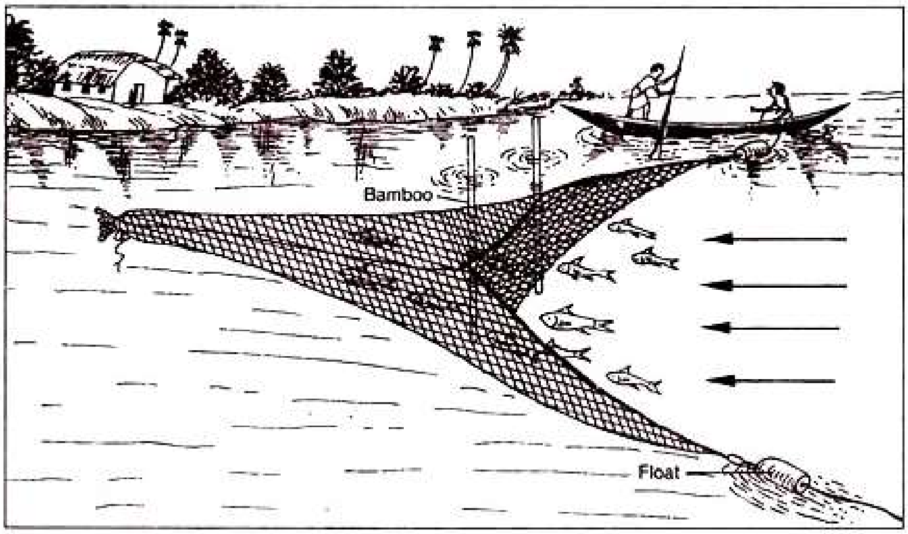
(xi) Fixed or Stationary Net

These nets are used to catch fish at inshore water during low tides. These nets are kept fixed with the help of floats, sinkers and stakes. It is rectangular or conical in shape. They are available in various sizes.

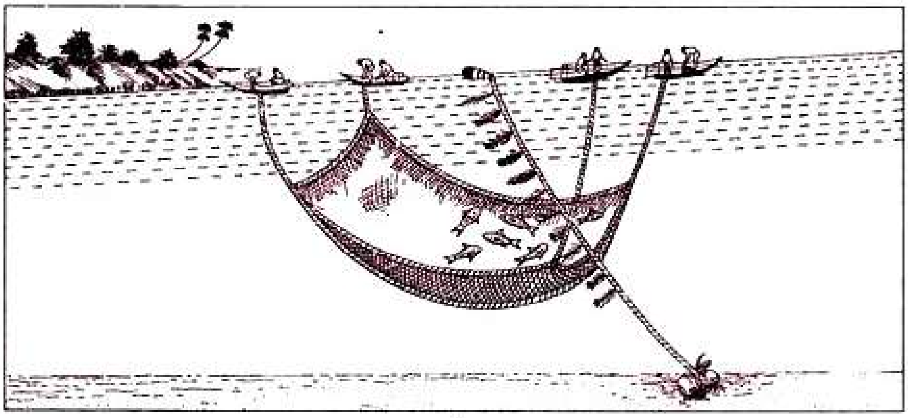
In West Bengal and Orissa conical fixed nets are used, which are called Ghurni Jal, or behundi, Kathia-kool Jal, Panch-Kathiaber Jal and Panch. However, rectangle nets used are called as Mai Jal in West Bengal, Barnada Jal in Orissa and Kakavalai, Jadi or Mtagh Jal in Tanjore.

(xii) Bag Nets

It is conical in shape without wings. Some commonly used bag nets are iroga in Andhra, thuriwala in Tamil Nadu and koliwala in Kerela. These nets are used with the help of two catamarans or boats. In the coasts of Mumbai and Gujarat a special type of bag net called 'dol' is used. It is conical with wide mouth. The mouth is fixed on a bamboo.



a



b

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(xiii) Scoop Net or Dip Net

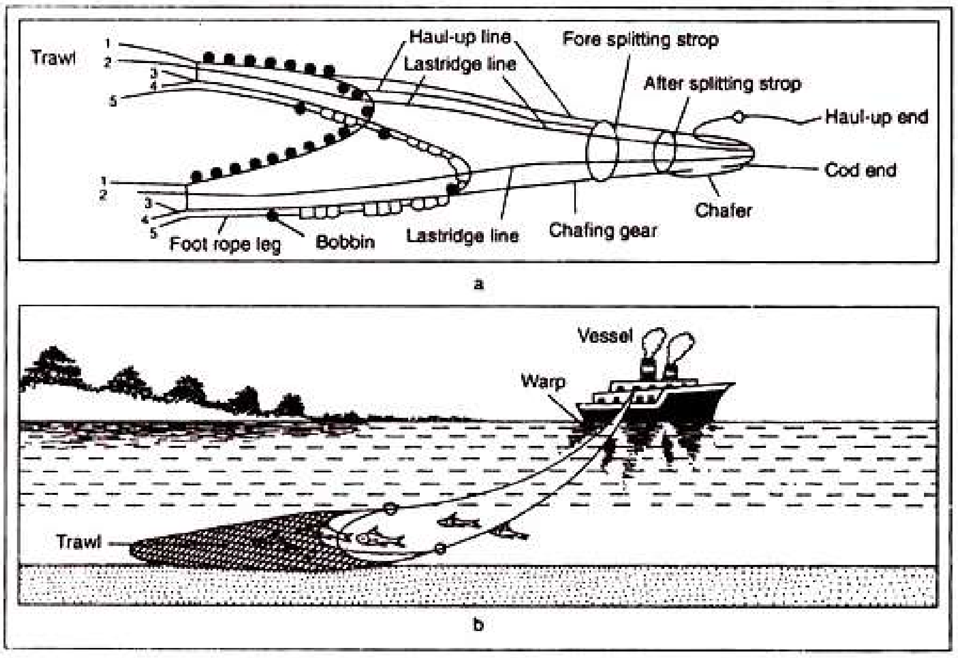
It is round in shape and is used to capture delicate fishes. It is like a finger bowl and can be moved swiftly in a scooping manner, collecting the fish.

(xiv) Hooks and Lines

It consists of two types of hand lines and long lines. Various types of hooks are used, such as chain hooks, baited hooks, revolving and non-revolving hooks for capture of larger fishes.

(xv) Trawls

These are large dragging type nets. There may be two types of trawls with beam called beam trawls and otter trawls.



B. Inland Fishing Crafts and Gears

Inland Fishing Crafts: Rafts and Dongas are age-old inland fishing crafts used in steady waters.

Rafts: Rafts are traditional crafts made by different types of materials. In Bihar earthen pots are tied together to provide support to a high bamboo platform. In West Bengal and Tamil Nadu stems of banana trees are held together to construct a floating platform.

In olden days buffalo skins are tied together to make a crude raft. In West Bengal a simple types of dugout canoe called Dongas are used. It is constructed by hollowing the stem of palm tree. It is commonly used for fishing in shallow waters. Vellum is stronger dugout canoe used in brackish waters of Kerala. In Tanjore and Tiruchirapalli chatty rafts made by earthenware are commonly used.

Types of Boats

Following types of boats are used for fishing in rivers:

i. Plank-Built Boat

These boats are sturdy and are used for fishing in rivers with strong tides and currents. In different regions different types of plank boats are operated. One of common types is 'Dinghis' used in west Bengal. Dinghi is narrow and it has a tapering bow and stern. It has no Keel. Dinghi is generally used to operate purse nets and dip nets. Another type is Chandi nauka, which is used to operate drift nets. It is 18 m long and 3 m wide. In Calcutta (Kolkata) and the other parts of West Bengal a medium sized boat called Mechho bachari is used to transport live fishes. However, in Chilka lake (brackish water lagoon) another type of plank boat called Nava is used.

ii. Kulnawa

It is a specialized boat used in the river Ganges for fishing of minnows. Kulnawa means open wale boat. This boat is used in calm water during night from February to April. It is brought from Tarai parts of Nepal.

Kulnawa is made up of 3 parts, viz., a frilled pole, screen platform and boat with one of the walls open. It is made by Sal wood or Kathal wood. It is 7 m long and is dinghy type of boat without any keel. The boat is painted by coaltar to keep it dark in colour.

The screen platform is made by 5 m x 0.5 m bamboo splinters, which are woven interlacing plastic cords. Screen is painted with enamel paint. The free margin of screen is always immersed in water. The frilled pole is 4-6 m long bamboo pole kept drooping as frill. 6% dry grass bunches are tied to these poles.

Inland Fishing Gears

India has a wide variety of diversified water bodies, hence the nets used are also diversified.

Nets used in Hill Streams

Cast nets are used in small pockets of hilly regions. Different types of traps and cast nets are fixed in narrow gaps of streams. It catches fish during breeding seasons.

Nets used in Ponds and Lakes

These are most commonly used for commercial fishing. Seines are large nets operated from boats. In big lakes and the Ganges the most commonly used seine is Jagat Ber Mahajal. Simple drag nets are also employed in ponds for commercial fishing.

Rangoon Nets and Uduvalai

They are used in those lakes where use of seines and dragnets are not easy. Rangoon net is made by fine cotton rectangular pieces of net. These pieces are tied in such a way so as to form a big wall. It is then spread in water with the help of floats.

The fish gets entangled in the net. Rangoon net is generally used for fishing in less deep water. However, in deep water bodies the use of uduvalai is preferred. It has small sinkers with footrope.

Gears used in Rivers

The following gears/ nets of different dimensions are used for fishing in rivers.

Seine and Drag Nets: They are most commonly used nets for fishing in rivers. Seines can be operated from one or more boats.

Kuriar Nets: It is used in shallow water for fishing of carps and herring (***Hilsa ilisha***). This net is very easy to operate. Kurian net is umbrella like and kept in inverted position and dipped in water for some times. Then it is pulled out with capture.

Kona Jal or Bhasa Gulli: It is a special type of large cotton seine net used for fishing of Hilsa.

Kona Jal: It is a special type of seine net of about 90 m x 9 m in size. It has conical pockets of small mesh size. These pockets are fixed at a distance of 8-10 m all along the net. It is made by cotton, fish once caught, cannot escape because of a valve-like flap present in the pocket. It is also called Bhasa Gulli.

Moi or Moia Jal: It is a simple net commonly used in shallow waters for fishing.

Jagat Ber or Maha Jal: It is a simple net used all around in rivers.

Chunti Jal: It is a type of drag net used in rivers of Bihar. Two fishermen operate it.

Kharra Jal: Hela jal, bhesal jal and firki jal are also commonly used for fishing of carps and Catla in the river Narmada.

Khorsula Jal or Koila Jal: It is a special type of dip net. Bhil tribal fishermen use this net in Narmada River for fishing of migratory Hilsa.

Jhanda Net: It is like an open bag operated in shallow waters. It is rectangular in shape with small mesh. This net is stretched on a bamboo frame.

Suti Jal: It is a long-tube like stationary bag net. It has a long wing.

Bada Jal: It is a modified type of Suti jal, which has a wide opening, being kept open with the help of a rod.

Purse Net: Special types of purse nets used for fishing in rivers are kharki jal and shanglo jal or sharki jal. In shanglo jal the mouth of purse can be opened or closed

with a vertical cord. However, in kharki jal a vertical bamboo rod is fixed to the lower part of mouth of the purse to open or close it. These nets are operated from a dug-out canoe.

C. Mechanized Craft

Selection of Gear: Selection of appropriate gear for catching fish is of utmost importance in order to increase catch.

Correct gear may be chosen taking into consideration of the following points:

1. For catching the big and strong fish, the gear should also be strong and sturdy with proper mash size.
2. To catch fish at various level of the water body, different nets should be used, viz. surface gill nets, column gill nets and bottom gill nets.
3. Fishes which swim in shoals, may be caught by using encircling nets like drag nets, purse nets, etc. Similarly using hooks and lines can catch the individual fish.

Maintenance of Gears

Proper care and handling of fishing gears after their use is as important as their use. Proper maintenance increases the durability of the gears.

Following precautions to be taken care of:

1. The gear should be washed thoroughly with the clean water and weeds and mud, etc. should be removed carefully.
2. Then dip the net in dilute $KMnO_4$ or $CuSO_4$ or common salt solution to get rid of harmful bacteria.
3. Wash again with clean water and then spread in shade for drying.
4. To increase durability and strength of the fibre of gear, it may be kept immersed for 10-15 min in hot tar diluted with kerosene.

D. Some Modern Crafts and Gears

Fishing technology continued to develop throughout history, employing improved and larger ships, more sophisticated fishing equipment, and various food preservation methods. Commercial fishing is now carried on in all types of waters, in all parts of the world, except where impeded by depth or dangerous currents or prohibited by law. Commercial fishing can be done in a simple manner with small vessels, little technical equipment, and little or no mechanization as in small local, traditional, or artisanal fisheries. It can also be done on a large scale with powerful deep-sea vessels and sophisticated mechanical equipment. Following are some of the modern commercial fishing technologies.

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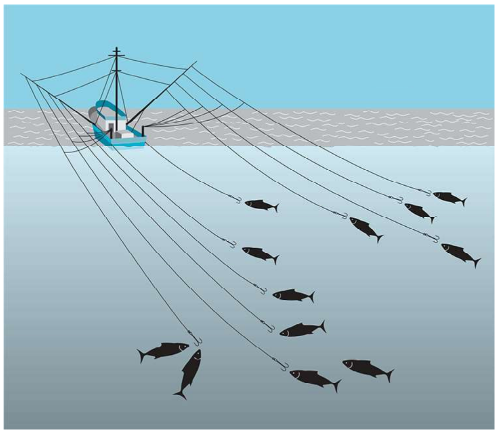
Commercial Fishing in Sea



Commercial Salmon Fishing Boat

Trolling Lines

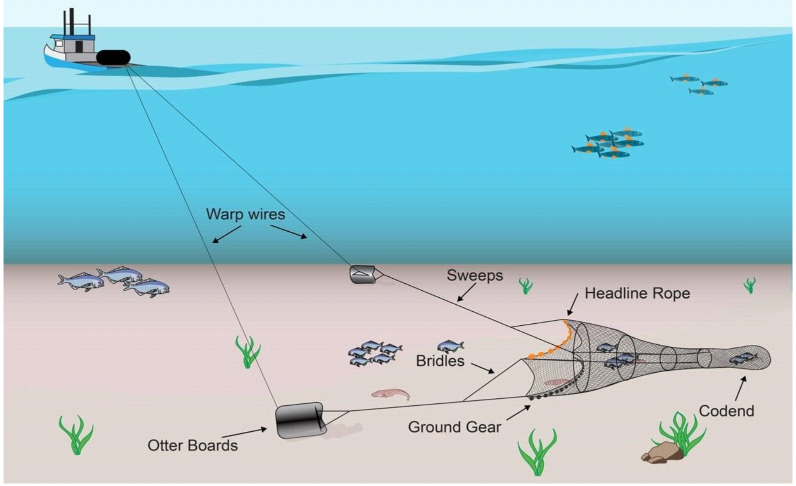
Trolling lines are simple hooked lines that are trailed from a moving vessel at a controlled depth. Bait may be artificial or natural and attracts predator fish that see what appears to be a smaller fish thrashing and turning in the water. The lure may be nothing more than a colourful piece of cloth, a small bunch of feathers, or a piece of skin from the bait fish, but it must be carefully adapted to local conditions and fish species and size. The use of outriggers can increase the number of lines that can be trolled and helps keep them from becoming tangled.



Modern Trawls

Trawl net is conical bag nets with two wings and codend where the catch is concentrated, performed by towing from one or two boats. The size of a trawl net may be defined by the length of the foot rope, headline or the number of meshes around the fishing circle, and the mesh size at that point. Mesh sizes are usually at their largest in the mouth of the trawl and progressively reduce towards the codend. Based on the operational position it is categorized into bottom trawl, mid water trawl or pelagic trawl.

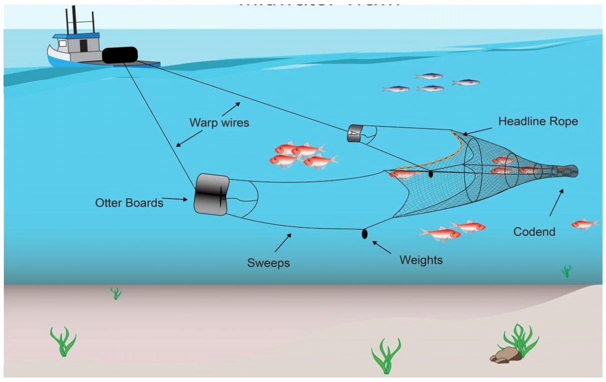
Bottom Trawling: When a trawl net is dragged in the bottom or just above the bottom, it is known as bottom trawling.



Mid Water Trawling

Mid water trawls are used for pelagic trawling. These are conical bags with a wide mouth made of light netting yarns and large meshes. Based on the construction of the trawls, they can be grouped into,

- Two Seam Pelagic Trawls.
- Four Seam Trawl - All the four equal panels for pair trawling and the upper and lower panels are identical, and the two identical side panels are narrow.



ANIMAL BIOTECHNOLOGY

1. ISOLATION OF GENOMIC DNA.

Isolation of DNA are done one large scale procedure which will yield DNA in excess of 200 kb and small scale procedure will develop DNA of 50-150 kb. These methods deals with DNA suitable for restriction enzyme digestion, genomic library construction, Southern blot analysis and PCR.

Large Scale Procedure

It generates 200-300 ug of DNA from 50 to 100mg of cells (c.a. 5 × 107 cells), grown in monolayer or suspension cultures, or from about 1-2 mg of solid tissue. The first procedure is a "hybrid" between the phenol and chloroform extraction methods, with the addition of Proteinase K digestion.

- Cells are washed in PBS and resuspended in cell lysis buffer containing Sarcosyl, EDTA and NaCl.
- Final concentrations of Sarcosyl, EDTA and NaCI are 1.2%, 0.1 M and 0.12 M, respectively.
- The majority of proteins are removed by Proteinase K treatment. The Proteinase K concentration used is very high (1000 ug/rnl) to shorten the time of the treatment, and the volume is small (about 10 ml), to decrease the cost of treatment.
- RNA is removed with RNase A and RNase T1 treatment after lowering the salt concentration below 0.1 M, to optimize RNase activity (see Chapter 1for a detailed explanation).
- Remaining proteins and RNases are removed by One Phenol : CIA Extraction.

The large scale method yields 50% to 80% high molecular weight DNA (I50-200 Kb) that is relatively devoid of protein and RNA contamination. The procedure works with most animal cells and can be easily scaled up by a factor of ten. It can be also scaled down for isolating DNA from as little as 5 mg of cells in microfuge tubes. The final yield is about 30-40 llg of DNA, an amount sufficient for several Southern blots.

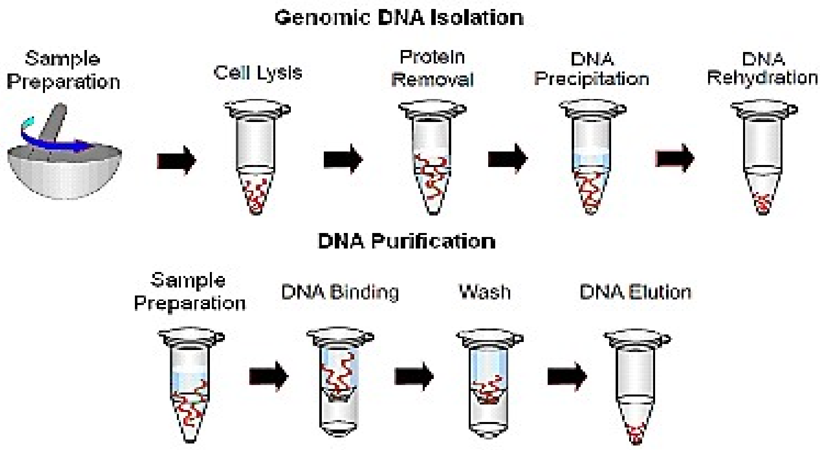
A disadvantage of the procedure is the usage of phenol and chloroform, which is risky for health and must be handled with care. It is also costly to dispose of these solvents. The protocol involves one day's work and contains at least one stopping point, where the protocol can be interrupted for one or more days.

Small Scale Procedure

- First method quickly isolates DNA from a small amount of cells in a single step. This method uses xanthogenate salt (Ihingan, 1992) and takes time about 1 hour. Moreover, the actual time spent on the procedure is less than

15 minutes. Thus, this method is ideal for isolation of DNA from multiple samples for Southern blot analysis and PCR.

- The second small scale procedure uses recently introduced DNAzol® solution for single step DNAisolation (Chomczynski *et al.*, 1997). The method can be finished in less than 30 minutes and results in isolation of 90% of DNA from cell or tissue material. The isolated DNA can contain partially degraded RNA that if necessary can be removed by RNase digestion. The reagent is commercially available from several sources. The DNA purified by this method from some sources was difficult to PCR without additional reprecipitation or purification.



2. ESTIMATION OF DNA

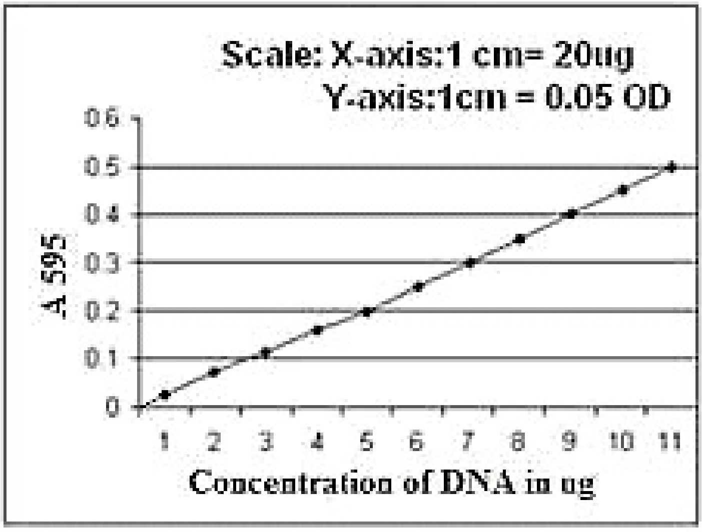
Principle: When DNA is treated with diphenylamine under the acidic condition a bluish green colored complex is formed which has an absorption peak at 595nm.This reaction is given by 2 deoxypentose in general. In acidic solution deoxypentose are converted into a highly reactive β hydroxyl leavulinic aldehyde which reacts with diphenylamine gives bluish green colored complex. The colour intensity was measured using a red filter at 595nm.

Reagent Required

1. Stock Standard Solution: 50mg of DNA was dissolved in 50ml of Saline Sodium Citrate buffer. Concentration 1mg/ml.
2. Working Standard Solution: 5ml of stock solution was diluted to 50ml with distilled water. concentration 100µg\ml.
3. Diphenylamine Reagent: 10g of pure diphenylamine was dissolved with 25ml of concentration sulphuric acid which was made up to 1ml with glacial acidic acid the solution must be prepared freshly.
4. Buffered Saline pH 7.4: 0.14N Sodium chloride and 0.02M sodium citrate.
5. Unknown Solution: The given unknown solution is mad up to 100ml with distilled water.

Procedure

1. 0.5-2.5ml of working standard solution is pipetted out into 5 test tubes labeled as s1- s5 where concentration ranging from 50-250µg.
2. 1ml and 2ml of unknown solution is pipetted out into two test tube u1 and u2.
3. The volume in all test tubes is made up to 3ml with distilled water and 3ml of distilled water alone serve as a blank.
4. 4ml of diphenylamine reagent was added to all the tubes. The tubes were kept in a boiling water bath at 36°C for 20 min. The tubes were than cooled and the bluish color developed is read at 595nm.
5. A standard graph is drawn taking concentration of DNA on x-axis and absorption of y-axis. From the standard graph the amount of DNA present in the unknown solution is calculated.



3. DEMONSTRATION OF ELISA

An Enzyme-Linked Immuno-Sorbent Assay (ELISA) is used to detect the presence of an antigen in a sample. The antigen is immobilized to the well of a plate by adsorption, or captured with a bound, antigen-specific antibody. A detection antibody is then added forming a complex with the antigen, if present. The detection antibody can be covalently linked to an enzyme, or itself be detected by a secondary, enzyme linked antibody. Enzyme substrate is then added to the wells producing a visible signal that is correlated with the amount of antigen and measured by a spectrophotometer.

Procedure:

1. 100µl peptide (@4µg/ml) in coating buffer is added to individual wells of a microtiter plate. Incubate the plate for 2 hours at 37°C or overnight at 4°C.

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2. Remove the coating solution and wash the plate three times by filling the wells with 100 µl PBS-0.05%Tween20. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.
3. Block the remaining protein-binding sites in the coated wells by adding 100µl blocking buffer, 3% skim milk in PBS per well. Incubate for 1 hour at RT with gentle shaking.
4. Wash the plate three times with 100ul PBS-0.05% Tween 20.
5. Add 50µl of diluted antibody to each well. Incubate the plate at 37! for an hour with gentle shaking.
6. Wash the plate six times with 100ul PBS-0.05% Tween 20.
7. Add 50µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Incubate at 37! for an hour.
8. Wash the plate six times with 100ul PBS-0.05% Tween20.
9. Prepare the substrate solution by mixing acetic acid, TMB and 0.03% H_2O_2 with the volume ratio of 4:1:5.
10. Dispense 50µl of the substrate solution per well with a multichannel pipe. Incubate the plate at 37! in dark for 15-30 mins.
11. After sufficient color development, add 100µl of stop solution to the wells (if necessary).
12. Read the absorbance (optical density at 450nm) of each well with a plate reader.

4. RAPD AND RFLP

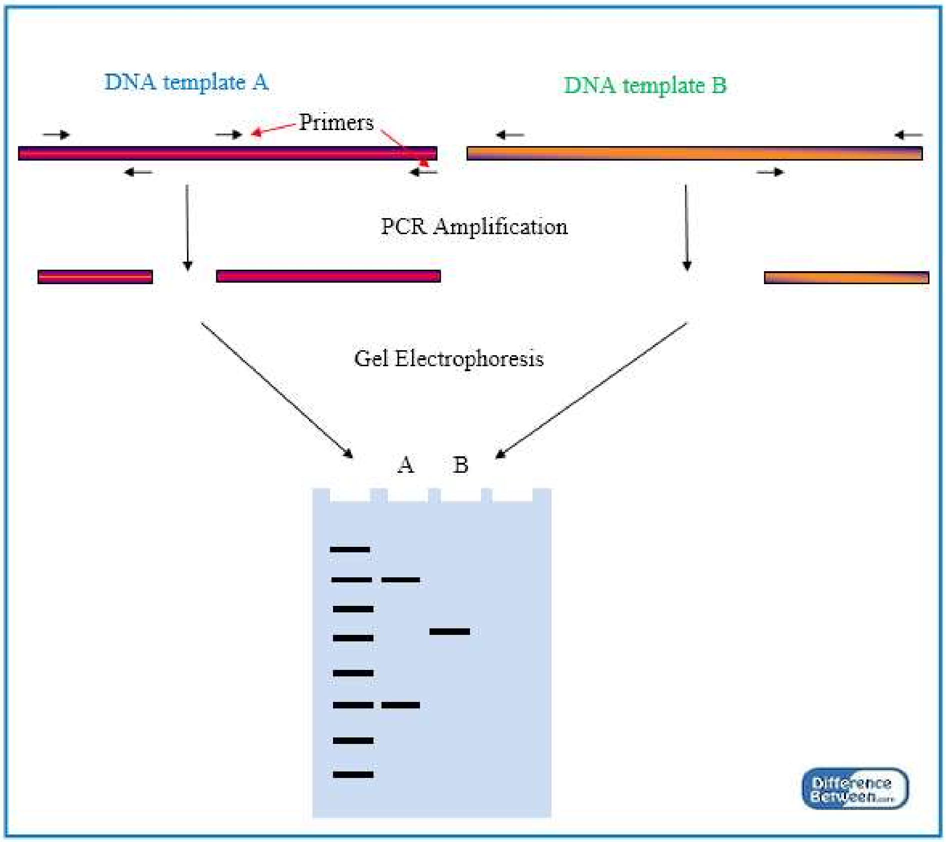
The main difference between RAPD and RFLP is that RAPD is a type of PCR which amplifies random fragments of DNA in a large template by using short primers whereas, in RFLP, one or more restriction enzymes digest the DNA sample, producing restriction fragments then separated by gel electrophoresis. Therefore, RADP only requires a small amount of DNA for the assay while RFLP requires a large amount of DNA. Furthermore, RAPD is a rapid process, which can detect 1-10 loci while RFLP is a slower process, which can only detect 1-3 loci. In addition, RAPD is unable to detect allelic variants while RFLP can detect allelic variants.

RAPD (Random Amplified Polymorphic DNA) and RFLP (Restriction Fragment Length Polymorphism) are two techniques in molecular biology to detect genetic markers, which are short DNA sequences, exploiting variations in homologous DNA.

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Similarities between RAPD and RFLP

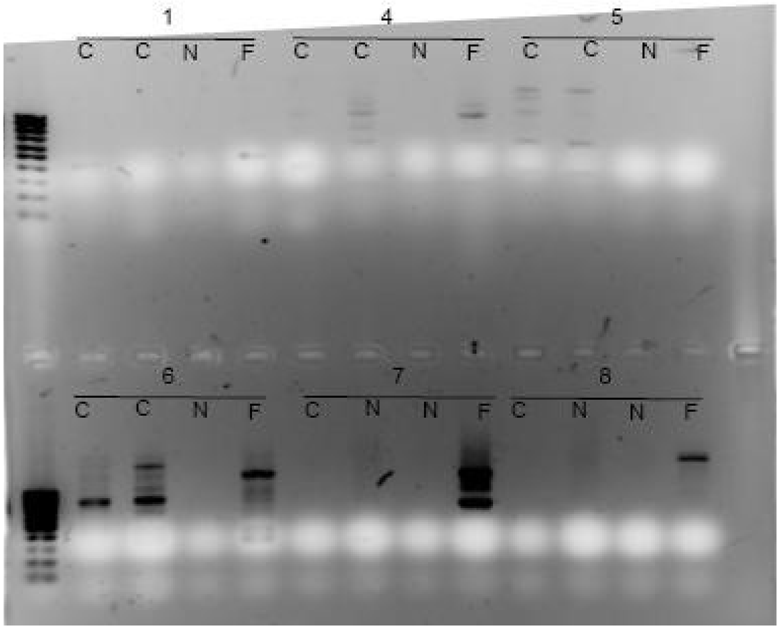
- RAPD and RFLP are two techniques in molecular biology to detect genetic markers, which exploit variation in homologous DNA.
- They detect DNA polymorphism required for genetic mapping, genome fingerprinting, and for the investigation of genetic relatedness.
- Therefore, they help to distinguish individuals, species or populations.
- Moreover, both methods use the total amount of DNA in the genome for the analysis.



What is RAPD?

RAPD or random amplified polymorphic DNA is a fast, PCR-based method for the detection of DNA variation. Two laboratories (Williams *et. al.*, 1990; Welsh and McClelland, 1990) independently developed the technique. Moreover, RAPD uses a single, arbitrary primer for the amplification of many discrete DNA products. The steps involved in RAPD are as follows:

1. DNA Extraction
2. Amplification by Random Primers
3. Gel electrophoresis and Visualization of Markers



Furthermore, by resolving the resulting patterns, a semi-unique DNA profile of a particular DNA sample can be generated. Due to the less specificity of the random primer used in the assay, comparatively a higher number of loci can be amplified per random primer. However, RAPD is an important technique for gene mapping, population genetics, molecular evolutionary genetics, animal and plant breeding, etc. Moreover, it is vital for beginners by means of speed, cost, efficiency as well as the number of markers generated.

What is RFLP?

Restriction Fragment Length Polymorphism (RFLP) is a method which exploits variation in homologous DNA. It is also a well-known method for its discrimination power. However, since RFLP is a non-PCR-based method, it uses restriction enzymes for the generation of fragments of the genome. Furthermore, RFLP detects the variations of lengths of restriction fragments among individuals. The three steps involved in RFLP are as follows:

1. Restriction Digestion of DNA
2. Gel Electrophoresis
3. Southern Blotting by Specific Probes and Detection

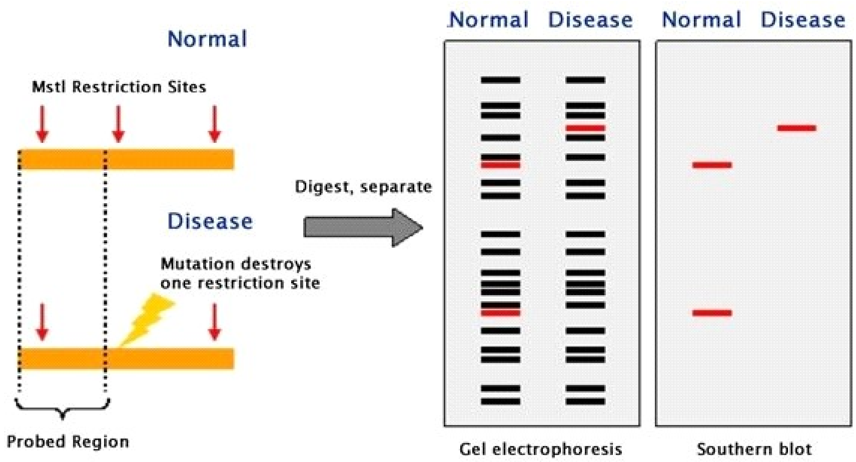
Restriction Fragment Length Polymorphism (RFLP) Technique

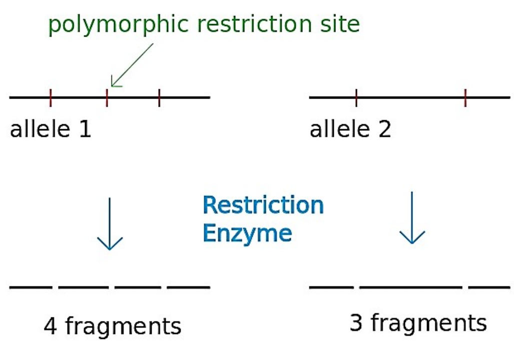
Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA.

- It is a technique that exploits variations in homologous DNA sequences.
- A restriction fragment length polymorphism is defined by the existence of alternative alleles associated with restriction fragments that differ in size

from each other. Simply, the variations in the restriction DNA fragments length between individuals of a species is called RFLP.

- The basic technique of identifying such restriction fragment length polymorphisms involve fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest.
- The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe.
- An RFLP occurs when the length of a detected fragment varies between individuals.
- Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to seek widespread application.





Moreover, restriction enzymes used in the process are unique for a particular DNA sequence. When the recognition sequence of the restriction enzymes is shorter, a greater number of fragments can be generated. Moreover, RFLP is important in

genotyping, forensics, paternity testing, the detection of patterns in hereditary diseases, and in the detection of disease carriers. However, it is a labour-intensive and time-consuming process, which requires a large amount of DNA.

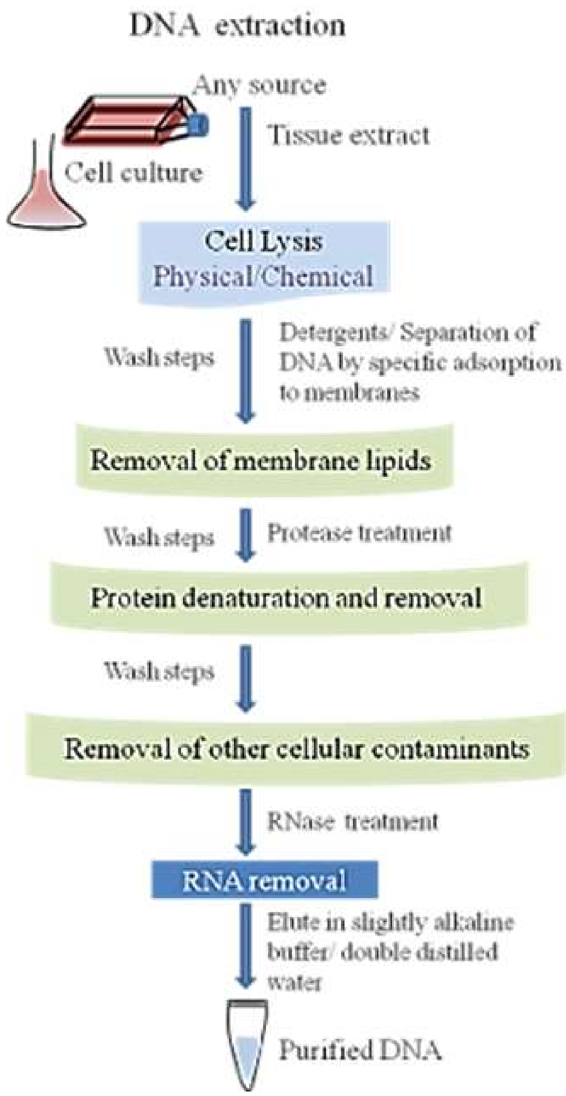
5. EXTRACTION AND PURIFICATION OF PLASMID DNA

DNA extraction is required for a variety of molecular biology applications. Many commercial kits are available to isolate DNA from a variety of biological materials. The sensitivity of Polymerase Chain Reaction (PCR) detection has been shown to be different for various DNA kits. Therefore, selecting the best methodology for your application is crucial.

Factors to be considered for selecting a kit include:

1. Sample Origin: Different kits are used to extract material from specific sources, including human tissues, blood, hair, rodent tissues, leaf tissue, bacteria, yeast, fungi, insect, stool, body fluids, spores, soil, clinical samples (e.g., biopsy samples, fine needle aspirates), forensic samples (e.g., dried blood spots, buccal swabs), and fingerprints.
2. Preparation Method: Sample preparations can be: fresh or previously frozen cell pellets, paraffin-embedded or formalin-fixed tissue sections, frozen tissue sections, ethanol-fixed cells, Oragene®-preserved samples, and samples from forensic sources which might contain very limited material.
3. Intended Use: The quality and purity of the DNA provided by the kit should be suitable for the intended downstream application, which could be sequencing, fingerprinting, PCR, quantitative PCR (qPCR), Southern blotting, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Restriction Fragment Length Polymorphism (RFLP) applications, restriction endonuclease digestion, or the preparation of shotgun libraries.
4. Humic Content: If the sample has humic content such as compost, sediment and manure, a kit/method that removes humic substances should be used, as they can inhibit downstream applications like PCR.
5. Sample Quantity: The kit to be used depends on the size of the sample being analysed. For example, the number of cultured mammalian cells (105-107) and bacterial cells (106-1011), the weight of human tissue, plant tissue or soil, the volume of blood, or even trace DNA samples from a crime scene.
6. Yield: the desired or expected amount of DNA to be purified from the sample. This is dependent upon the sample as well as the downstream applications.
7. Simplicity: The kit operation depends on the experience of the user, and the degree of control desired over each stage of the sample processing.

NOTES



The basic criteria that any method of DNA isolation from any sample type should meet include:

(1) Efficient extraction of DNA from the sample.

(2) Production of a sufficient amount of DNA for use in downstream processes.

(3) Successful removal of contaminants.

(4) Isolation of high quality and high purity DNA.

Assays to Assess Sample Purity and Quality Control

Ultraviolet absorbance can be used to assess the purity of the extracted DNA. For a pure DNA sample, the ratio of absorbance at 260 nm and absorbance at 280 nm (A260/A280) is 1.8. A ratio of < 1.8 indicates the sample is contaminated with protein or an organic solvent such as phenol, often used during extraction processes. The quantification of double-stranded DNA can also be assessed by the Qubit assay, which relies on the principle of DNA-selective fluorescent dyes

although it may underestimate in DNA extracted after RNA extraction with Trizol. DNA quality can be assessed by visualization on agarose gels.

Common DNA Extraction Methods

Different extraction methods result in different yields and purity of DNA. Some of the extraction methods have been systematically evaluated for specific applications such as soil and sediment samples, human microbiome, and fecal samples.

Organic Extraction

In this method, cells are lysed and cell debris is usually removed by centrifugation. Then, proteins are denatured/digested using a protease, and precipitated with organic solvents such as phenol, or 1:1 mixture of phenol and chloroform. The protein precipitate is removed following separation by centrifugation. Purified DNA is usually recovered by precipitation using ethanol or isopropanol. At some point in the process, RNAs are degraded through incubation with RNase. In the presence of monovalent cations such as Na^+, and at -20°C, absolute ethanol efficiently precipitates polymeric nucleic acids and leaves behind short-chain and monomeric nucleic acid components, including the ribonucleotides from RNase treatment in solution. This method uses hazardous organic solvents, is relatively time-consuming, and residual phenol or chloroform may affect downstream applications such as PCR. An example of a commercially available kit that relies on this chemistry is the Easy-DNA® Kit from Thermo Fisher.

Silica-Based Technology

Silica-based technologies are widely employed in current kits. DNA adsorbs specifically to silica membranes/beads/particles in the presence of certain salts and at a defined pH. The cellular contaminants are removed by wash steps. DNA is eluted in a low salt buffer or elution buffer. Chaotropic salts are included in the kit buffers to aid in protein denaturation and extraction of DNA. This method can be incorporated in spin columns and microchips, is cost-effective, has a simpler and faster procedure than the organic extraction, and is suitable for automation. Kits based on this method include Purelink Genomic DNA extraction kit from Thermo Fisher and DNeasy Blood and Tissue Kit from QIAGEN.

Magnetic Separation

Magnetic separation is based on DNA reversibly binding to a magnetic solid surface/bead/particles that have been coated with a DNA binding antibody, or a functional group that interacts specifically with DNA. After DNA binding, beads are separated from other contaminating cellular components, washed, and the purified DNA is eluted using ethanol extraction. This method is rapid, simple to perform and can be automated. However, it can be more costly than other methodologies. Examples of commercially available kits include the Agencourt DNA advance Kit from Beckman Coulter and Magnetic Beads Genomic DNA Extraction Kit from Geneaid.

Anion Exchange Technology

DNA extraction by anion exchange chromatography is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using a low or medium salt buffer, and purified DNA is eluted using a high salt buffer. This technology is most commonly employed in plasmid isolation kits such as PureLink® HiPure Plasmid DNA Purification Kits from Thermo Fisher, QIAGEN plasmid mini/midi kits and Genomic-tip, and NucleoBond® PC kits from Macherey Nagel.

DNA Isolation from Microbes

Bacterial cells are cultured in liquid media until they reach a maximum density of 2-3x10^9 cells/ml, and then harvested. The collected cells are lysed, often done chemically, using reagents such as lysozyme, EDTA, lysozyme and EDTA and other detergents, etc. Cellular components are then removed using one of the above listed technologies, for example organic extraction or silica-based technologies. The final step involves DNA precipitation to obtain pure DNA at a high concentration. This procedure can be applied to a wide variety of microbes and other unicellular organisms such as yeast.

DNA Extraction from Animal Cells and Tissues

The basic steps involved in extracting DNA from animal cells and tissues is the same as discussed for microbes. However, kits need to incorporate modifications to take into account the special features of animal cells. Culturing and preparing of animal cells is often very different from that of microbial cells. Animal cells do not have a cell wall like microbial cells, and consequently, are easier to lyse. Thus, they can be lysed using only detergents. However, when cells are part of intact animal tissue, the tissue needs to first be mechanically homogenized or treated with enzymes for lysis. Cell lysis is followed by the isolation and purification of DNA from other cellular components.

Many kits do not use the conventional organic extraction method requiring phenol/chloroform extraction and ethanol precipitation. This can be useful, as it minimizes damage to DNA by organic solvents. For example AccuPrep Genomic DNA Extraction (Bioneer) employ columns packed with glass fibers to extract the DNA. QIAamp DNA mini (QIAGEN) uses silica-membrane containing columns, which are able to retain DNA under specific pH and salt conditions. Agencourt DNAdvance (Beckman Coulter) is an example of a kit employing magnetic separation.

DNA Extraction from Plant Tissue and Cells

The basic steps used for DNA isolation require adaptations to make them suitable for the different characteristics of the plant cells and tissue. Chemicals or enzymes used to lyse microbial and mammalian cells may not be equally effective on plant

cells. For example, lysozyme is often included in kits to lyse bacterial cells but has no effect on plant cells due to the presence of the cell wall. Furthermore, the biochemical content of plant cells is very different from microorganisms and animal cells. Many plant species have a high content of polysaccharides and polyphenols which are not removed by phenol extraction (unlike microbes). Therefore, different methods and reagents need to be included in commercially available kits to address the special features of plant cells.

One method is to utilize a detergent called cetyltrimethylammonium bromide (CTAB) which forms an insoluble complex with nucleic acid and selectively precipitates DNA, leaving behind carbohydrates, proteins and other contaminating components. The DNA-containing precipitate can be decomplexed by dissolving it in NaCl. CTAB can be included in any step of the extraction process. To remove polyphenols higher concentration of CTAB with polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) can be employed.

Another method is to use guanidium thiocyanate (GITC), which assists DNA purification from plant materials in two ways. Firstly, it denatures and dissolves proteins, disintegrates cellular structures, and dissociates nucleoproteins from the nucleic acid. Due to this property, GITC can be used to release DNA from almost any type of tissue [43] Secondly, DNA binds strongly to silica particles in the presence of GITC. This property can be utilized to separate DNA from the denatured proteins and other biochemical or cellular components. Commonly, silica particles are packed in chromatography columns and a DNA extract treated with GITC is applied. DNA binds selectively to the column and can be eluted in the last step after washing away the cellular contaminants.

In some DNA extraction procedures, ascorbic acid, diethyldithiocarbamic acid and 2-mercaptoethanol might be included to protect DNA against oxidation and degradation. RNA can be removed by using RNase. The quality of the DNA isolated is largely dependent on the physiological condition of the plant material, rather than the kit protocol.

6. SPOTTER: MODELS OF PCR, SOUTHERN BLOTTING

Quantitative PCR (qPCR) is a workhorse laboratory technique for measuring the concentration of a target DNA sequence with high accuracy over a wide dynamic range. The gold standard method for estimating DNA concentrations via qPCR is quantification cycle (C_q) standard curve quantification, which requires the time- and labor-intensive construction of a C_q standard curve. In theory, the shape of a qPCR data curve can be used to directly quantify DNA concentration by fitting a model to data; however, current empirical model-based quantification methods are not as reliable as C_q standard curve quantification.

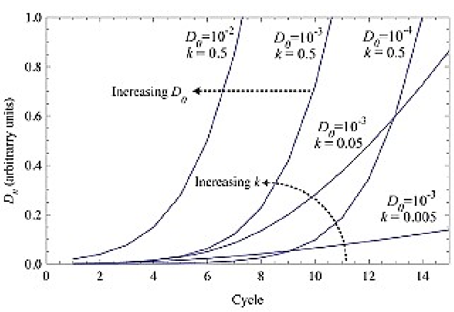
Principal Findings

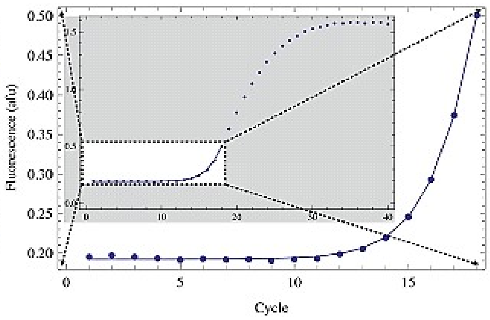
We have developed a two-parameter mass action kinetic model of PCR (MAK2) that can be fitted to qPCR data in order to quantify target concentration from a

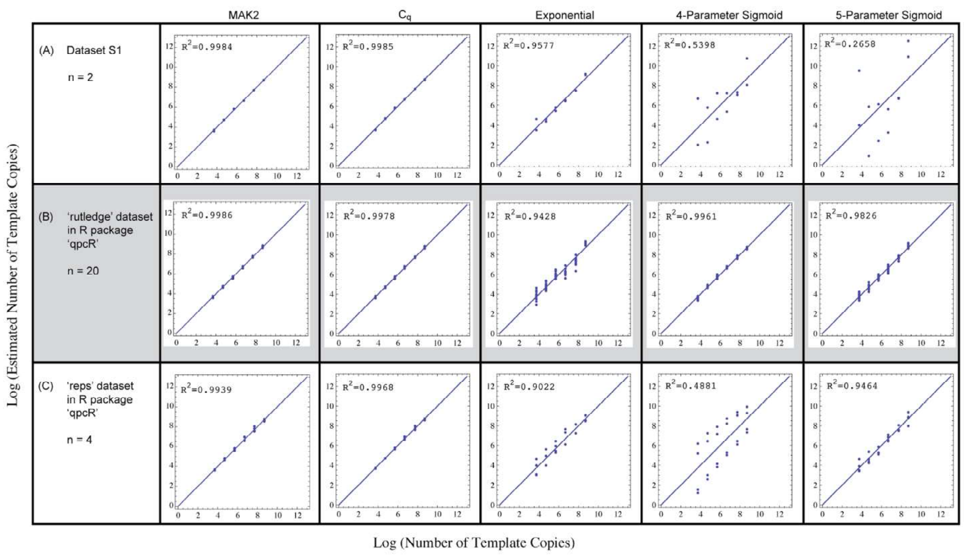
single qPCR assay. To compare the accuracy of MAK2-fitting to other qPCR quantification methods, we have applied quantification methods to qPCR dilution series data .generated in three independent laboratories using different target sequences. Quantification accuracy was assessed by analyzing the reliability of concentration predictions for targets at known concentrations. Our results indicate that quantification by MAK2-fitting is as reliable as C_q standard curve quantification for a variety of DNA targets and a wide range of concentrations.

Significance

We anticipate that MAK2 quantification will have a profound effect on the way qPCR experiments are designed and analyzed. In particular, MAK2 enables accurate quantification of portable qPCR assays with limited sample throughput, where construction of a standard curve is impractical.







Southern blotting is a laboratory technique used to detect a specific DNA sequence in a blood or tissue sample. A restriction enzyme is used to cut a sample of DNA into fragments that are separated using gel electrophoresis. The DNA fragments are transferred out of the gel to the surface of a membrane. The membrane is exposed to a DNA probe labeled with a radioactive or chemical tag. If the probe binds to the membrane, then the probe sequence is present in the sample.

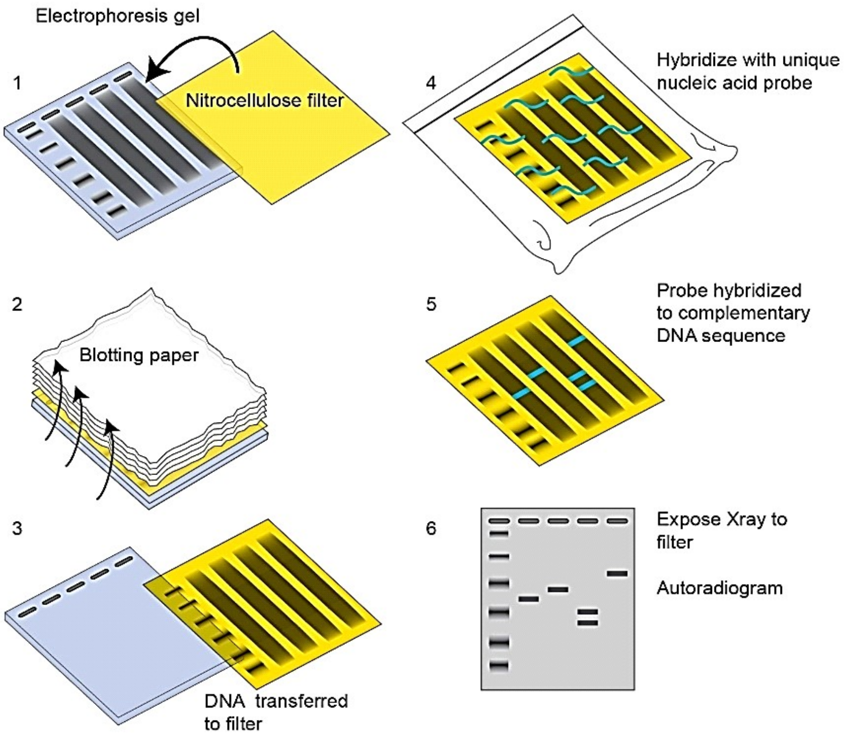
Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

The method is named after the British biologist Edwin Southern, who first published it in 1975. Other blotting methods (i.e., Western Blot, Northern Blot, Eastern Blot, Southwestern Blot) that employ similar practices, but using RNA or protein, have later been named in reference to Edwin Southern's name.

Method

1. Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments.
2. The DNA fragments are then electrophoresed on an agarose gel to separate them by size.
3. If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl. This depurinates the DNA fragments, breaking the DNA into smaller pieces, thereby allowing more efficient transfer from the gel to membrane.
4. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (typically containing sodium hydroxide) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged thymine residues of DNA to a positively charged amino groups of membrane, separating it into single DNA strands for later hybridization to the probe (see the figure below), and destroys any residual RNA that may still be present in the DNA. The choice of alkaline over neutral transfer methods, however, is often empirical and may result in equivalent results.
5. A sheet of nitrocellulose (or, alternatively, nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. If transferring by suction, 20X SSC buffer is used to ensure a seal and prevent drying of the gel. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA from the gel onto the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
6. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours (standard conditions; nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.

7. The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, the hybridization probe may be made from RNA, rather than DNA. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon or herring sperm DNA for blocking of the membrane membrane surface and target DNA, deionized formamide, and detergents, such as SDS to reduce non-specific binding of the probe.
8. After hybridization, excess probe is washed from the membrane (typically using SSC buffer), and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.



Result

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. It also allows for the fixation of the target-probe hybrids, required for analysis by autoradiography or other detection methods. Southern blots performed with

restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (for example, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

Applications

Southern blotting transfer may be used for homology-based cloning on the basis of amino acid sequence of the protein product of the target gene. Oligonucleotides are designed so that they are similar to the target sequence. The oligonucleotides are chemically synthesized, radiolabeled, and used to screen a DNA library, or other collections of cloned DNA fragments. Sequences that hybridize with the hybridization probe are further analysed, for example, to obtain the full length sequence of the targeted gene.

Southern blotting can also be used to identify methylated sites in particular genes. Particularly useful are the restriction nucleases MspI and HpaII, both of which recognize and cleave within the same sequence. However, HpaII requires that a C within that site be methylated, whereas MspI cleaves only DNA unmethylated at that site. Therefore, any methylated sites within a sequence analyzed with a particular probe will be cleaved by the former, but not the latter, enzyme.

7. CLONING VECTOR - IMAGES

A cloning vector is a small piece of DNA that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes. The cloning vector may be DNA taken from a virus, the cell of a higher organism, or it may be the plasmid of a bacterium. The vector therefore contains features that allow for the convenient insertion or removal of a DNA fragment to or from the vector, for example by treating the vector and the foreign DNA with a restriction enzyme that cuts the DNA. DNA fragments thus generated contain either blunt ends or overhangs known as sticky ends, and vector DNA and foreign DNA with compatible ends can then be joined together by molecular ligation. After a DNA fragment has been cloned into a cloning vector, it may be further subcloned into another vector designed for more specific use.

There are many types of cloning vectors, but the most commonly used ones are genetically engineered plasmids. Cloning is generally first performed using ***Escherichia coli***, and cloning vectors in ***Escherichia coli*** include plasmids, bacteriophages (such as, phage λ), cosmids, and Bacterial Artificial Chromosomes (BACs). Some DNA, however, cannot be stably maintained in E.

coli, for example very large DNA fragments, and other organisms such as yeast may be used. Cloning vectors in yeast include Yeast Artificial Chromosomes (YACs).

Features of a Cloning Vector

All commonly used cloning vectors in molecular biology have key features necessary for their function, such as a suitable cloning site and selectable marker. Others may have additional features specific to their use. For reason of ease and convenience, cloning is often performed using *Escherichia coli*. Thus, the cloning vectors used often have elements necessary for their propagation and maintenance in E. coli, such as a functional origin of replication (ori). The ColE1 origin of replication is found in many plasmids. Some vectors also include elements that allow them to be maintained in another organism in addition to *Escherichia coli*, and these vectors are called shuttle vector.

Cloning Site

All cloning vectors have features that allow a gene to be conveniently inserted into the vector or removed from it. This may be a Multiple Cloning Site (MCS) or polylinker, which contains many unique restriction sites. The restriction sites in the MCS are first cleaved by restriction enzymes, then a PCR-amplified target gene also digested with the same enzymes is ligated into the vectors using DNA ligase. The target DNA sequence can be inserted into the vector in a specific direction if so desired. The restriction sites may be further used for sub-cloning into another vector if necessary.

Other cloning vectors may use topoisomerase instead of ligase and cloning may be done more rapidly without the need for restriction digest of the vector or insert. In this TOPO cloning method a linearized vector is activated by attaching topoisomerase I to its ends, and this "TOPO-activated" vector may then accept a PCR product by ligating both the 5' ends of the PCR product, releasing the topoisomerase and forming a circular vector in the process. Another method of cloning without the use of DNA digest and ligase is by DNA recombination, for example as used in the Gateway cloning system. The gene, once cloned into the cloning vector (called entry clone in this method), may be conveniently introduced into a variety of expression vectors by recombination.

Selectable Marker

A selectable marker is carried by the vector to allow the selection of positively transformed cells. Antibiotic resistance is often used a s marker, an example being the beta-lactamase gene, which confers resistance to the penicillin group of beta-lactam antibiotics like ampicillin. Some vectors contain two selectable markers, for example the plasmid pACYC177 has both ampicillin and kanamycin resistance gene. Shuttle vector which is designed to be maintained in two different organisms may also require two selectable markers, although some selectable markers such as resistance to zeocin and hygromycin B are effective in different cell

types. Auxotrophic selection markers that allow an auxotrophic organism to grow in minimal growth medium may also be used; examples of these are LEU2 and URA3 which are used with their corresponding auxotrophic strains of yeast.

Another kind of selectable marker allows for the positive selection of plasmid with cloned gene. This may involve the use of a gene lethal to the host cells, such as barnase, Ccda, and the parD/parE toxins. This typically works by disrupting or removing the lethal gene during the cloning process, and unsuccessful clones where the lethal gene still remains intact would kill the host cells, therefore only successful clones are selected.

Reporter Gene

Reporter genes are used in some cloning vectors to facilitate the screening of successful clones by using features of these genes that allow successful clone to be easily identified. Such features present in cloning vectors may be the lacZα fragment for α complementation in blue-white selection, and/or marker gene or reporter genes in frame with and flanking the MCS to facilitate the production of fusion proteins. Examples of fusion partners that may be used for screening are the Green Fluorescent Protein (GFP) and luciferase.

Expression Vector

A cloning vector need not contain suitable elements for the expression of a cloned target gene, such as a promoter and ribosomal Binding Site (RBS), many however do, and may then work as an expression vector. The target DNA may be inserted into a site that is under the control of a particular promoter necessary for the expression of the target gene in the chosen host. Where the promoter is present, the expression of the gene is preferably tightly controlled and inducible so that proteins are only produced when required. Some commonly used promoters are the T7 and lac promoters. The presence of a promoter is necessary when screening techniques such as blue-white selection are used.

Cloning vectors without promoter and RBS for the cloned DNA sequence are sometimes used, for example when cloning genes whose products are toxic to E. coli cells. Promoter and RBS for the cloned DNA sequence are also unnecessary when first making a genomic or cDNA library of clones since the cloned genes are normally subcloned into a more appropriate expression vector if their expression is required.

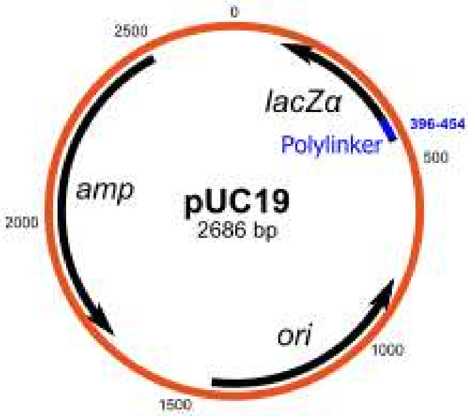
Some vectors are designed for transcription only with no heterologous protein expressed, for example for in vitro mRNA production. These vectors are called transcription vectors. They may lack the sequences necessary for polyadenylation and termination, therefore may not be used for protein production.

Types of Cloning Vectors

A large number of cloning vectors are available, and choosing the vector may depend upon a number of factors, such as the size of the insert, copy number and

cloning method. Large insert may not be stably maintained in a general cloning vector, especially for those with a high copy number, therefore cloning large fragments may require more specialized cloning vector.

NOTES



The PUC plasmid has a high copy number, contains a multiple cloning site (polylinker), a gene for ampicillin antibiotic selection, and can be used for blue-white screen.

Plasmid Vector

Plasmids are autonomously replicating circular extra-chromosomal DNA. They are the standard cloning vectors and the ones most commonly used. Most general plasmids may be used to clone DNA insert of up to 15 kb in size. One of the earliest commonly used cloning vectors is the pBR322 plasmid. Other cloning vectors include the PUC series of plasmids, and a large number of different cloning plasmid vectors are available. Many plasmids have high copy number, for example pUC19 which has a copy number of 500-700 copies per cell, and high copy number is useful as it produces greater yield of recombinant plasmid for subsequent manipulation. However low-copy-number plasmids may be preferably used in certain circumstances, for example, when the protein from the cloned gene is toxic to the cells.

Some plasmids contain an M13 bacteriophage origin of replication and may be used to generate single-stranded DNA. These are called phagemid, and examples are the p Blue script series of cloning vectors.

Bacteriophage

The bacteriophages used for cloning are the λ phage and M13 phage. There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 53 kb), therefore to allow foreign DNA to be inserted into phage DNA, phage cloning vectors may need to have some non-essential genes deleted, for example the genes for lysogeny since using phage λ as a cloning vector involves only the lytic cycle. There are two kinds of λ phage vectors - insertion vector and replacement vector. Insertion vectors contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted. In replacement vectors, the cleavage

sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted.

There is also a lower size limit for DNA that can be packed into a phage, and vector DNA that is too small cannot be properly packaged into the phage. This property can be used for selection - vector without insert may be too small, therefore only vectors with insert may be selected for propagation.

Cosmid

Cosmids are plasmids that incorporate a segment of bacteriophage λ DNA that has the cohesive end site (cos) which contains elements required for packaging DNA into λ particles. It is normally used to clone large DNA fragments between 28 and 45 Kb.

Bacterial Artificial Chromosome

Insert size of up to 350 kb can be cloned in bacterial Artificial Chromosome (BAC). BACs are maintained in ***Escherichia coli*** with a copy number of only 1 per cell. BACs are based on F plasmid, another artificial chromosome called the PAC is based on the P1 phage.

Yeast Artificial Chromosome (YAC)

Yeast artificial chromosome are used as vectors to clone DNA fragments of more than 1 mega base (1Mb=1000kb) in size. They are useful in cloning larger DNA fragments as required in mapping genomes such as in human genome project. It contains a telomeric sequence, an autonomously replicating sequence (features required to replicate linear chromosomes in yeast cells). These vectors also contain suitable restriction sites to clone foreign DNA as well as genes to be used as selectable markers.

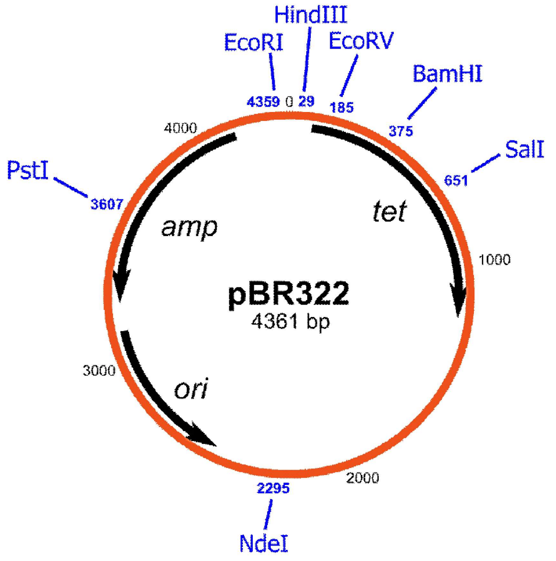
Human Artificial Chromosome

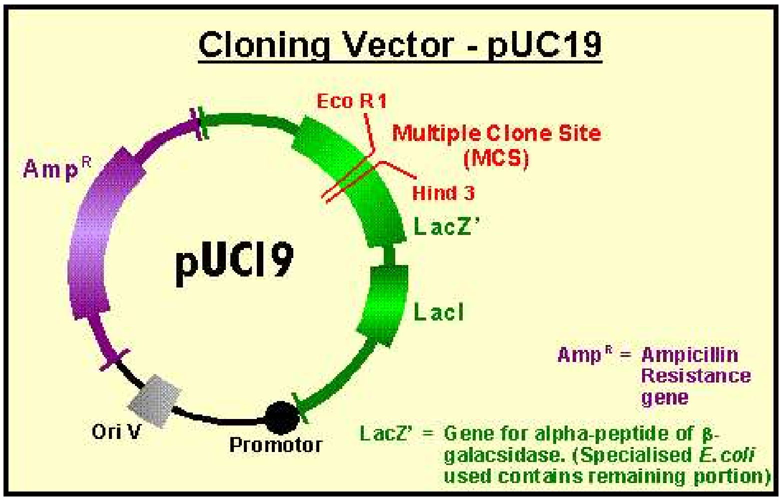
Human artificial chromosome may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function. It can carry very large DNA fragment (there is no upper limit on size for practical purposes), therefore it does not have the problem of limited cloning capacity of other vectors, and it also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

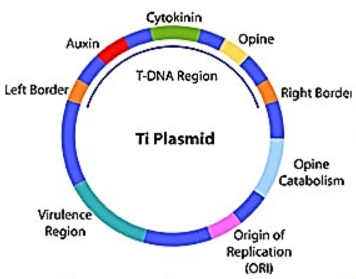
Animal and plant viral vectors Viruses that infect plant and animal cells have also been manipulated to introduce foreign genes into plant and animal cells. The natural ability of viruses to adsorb to cells, introduce their DNA and replicate have made them ideal vehicles to transfer foreign DNA into eukaryotic cells in culture. A vector based on Simian virus 40 (SV40) was used in first cloning experiment involving mammalian cells. A number of vectors based on other type of viruses like Adenoviruses and Papilloma virus have been used to clone genes in mammals. At present, retroviral vectors are popular for cloning genes in mammalian cells. In

case of plants like Cauliflower mosaic virus, Tobacco mosaic virus and Gemini viruses have been used with limited success.

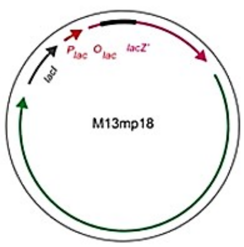
Images of Cloning Vector



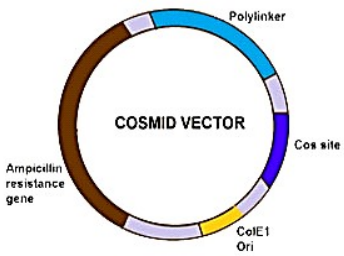




i) Plasmid Vector



ii) Bacteriophages Vector



iii) Cosmids

BIOPHYSICS, BIOSTATISTICS AND BIOINFORMATICS

1. SPECTROPHOTOMETER, pH METER AND ELECTROPHORETIC UNIT AS SPOTTERS

Determination of pH is one of the most frequently performed measurements in chemistry. The potentiometric method with a glass electrode has been widely used for pH measurements but has drawbacks, such as the need for a reference electrode, susceptibility to electrical interference, instrument drift, and the need for physical contact with the solution. It is desirable to have alternative methods for pH determination. One such method is spectrophotometric measurement with the use of a suitable pH indicator. In the spectrophotometric method used here, the pH of an unknown solution is determined by addition of a small amount of a pH indicator and determination of the extent of dissociation of the indicator (a weak acid). Because overlap exists between the spectra for the acid form (generically represented as Hln) and base form (In^-) of the indicator, it is necessary to determine individual molar absorptivities for each form at two wavelengths (λ_1 and λ_2). Usually these are the wavelength peaks (absorption maxima) of HIn and In^-. Assuming that the absorbances of the two forms are additive (independent of one another), we obtain two simultaneous linear equations for the absorption at the two wavelengths measured:

$$A_1 = \varepsilon_1^{HIn}\ b\ [HIn] + \varepsilon_1^{In-}\ b\ [In^-]$$

$$A_2 = \varepsilon_2^{HIn}\ b\ [Hin] + \varepsilon_2^{In-}\ b\ [In^-]$$

Where,

A_1 and A_2 are the absorbances at λ_1 and λ_2

ε_1^{HIn} and ε_2^{HIn} are the molar absorptivities of Hln at λ_1 and λ_2

ε_1^{In-} and ε_2^{In-} are the molar absorptivities of In^- at λ_1 and λ_2

The pH indicator Bromocresol Green ($Ka = 1.60 \times 10^{-5}$) will be used for the spectrophotometric procedure.

The pH of an acetate buffer will be determined by both spectrophotometric and potentiometric methods and the results will be compared.

Apparatus

1. Plastic Cuvette (1)
2. 100 mL Volumetric Flask (1)

3. 50 mL Volumetric Flasks (3)
4. 5 and 10 mL Pipets (1 each)

Instrumentation

1. WPA Biowave II or Comparable Benchtop UV-Visible Spectrophotometer
2. Digital pH meter and pH Electrode

Solutions Required

1. Standard pH Calibration Buffer Solutions (pH = 4.00 and pH = 7.00)
2. 1.0×10^{-4} M Bromocresol Green (Measure out about 75 mL with a Graduated Cylinder)
3. 0.10 M HCl
4. 0.10 M NaOH
5. 2.40 M Acetic Acid

Solution to be Prepared

Buffer Solution: Pipet 5.00 mL of 2.40 M acetic acid into a 100 mL volumetric flask and dilute with about 50 mL of deionized water. Weigh about 0.825 g (to 0.001 g) of sodium acetate ($NaC_2H_3O_2$, FW = 82.03) and quantitatively transfer to the same volumetric flask and dissolve completely. Finally, fill the flask to the mark with deionized water and mix thoroughly. Calculate and record the analytical concentrations of the acetic acid and acetate.

Procedure

(1) **Preparing the Sample Solutions for the Spectrophotometric Method:** Pipet 10 mL of the Bromocresol Green solution to each of three 50-mL volumetric flasks. Use a graduated cylinder to add 25 mL of 0.10 M HCl, 0.10 M NaOH, and the buffer solution, respectively. Dilute to the mark with deionized water and mix thoroughly.

(2) **Measuring the Baseline:** Rinse the cuvette with tap water and deionized water, fill it with deionized water, and place it in the holder. Set up the spectrophotometer and measure the 'Baseline' using the deionized water.

(3) **Measuring the Spectra of the Three Solutions:** Rinse the cuvette with a sample solution twice, fill it with the solution, place it in the holder, and measure the spectrum. Measure the spectra of all three solutions, i.e., acidic, basic, and buffer. If you wish, you can check for spectrometer drift or alignment problems by thoroughly rinsing the cuvette and re-measuring the spectrum of the deionized water (the blank).

(4) **Reading the Absorbance:** Locate the peak wavelengths at the absorption maxima of the acidic and basic solutions (i.e., for Hln and In^{-}). Write down the peak wavelengths in your notebook. Then read and record the absorbance at the "Two Peak Wavelengths for All Three Solutions".

Remember that the peak wavelengths of the buffer solution may be slightly different from the ones of the acidic and basic solutions, but have to read the absorbance of the buffer solution at the peak wavelengths of the acidic and basic solutions. Manually record enough wavelength points for the three spectra that you can reconstruct them in MS Excel or some other appropriate graphing program.

(5) **Measuring pH with a Glass Electrode:** Calibrate the pH meter with the two standard buffers - pH 7.00 and pH 4.00. Measure the pH of the unknown buffer solution.

2. CONSTRUCTION OF GRAPH AND BAR DIAGRAM USING BIOLOGICAL DATA.

A graph is a picture designed to express words, particularly the connection between two or more quantities. A simple graph typically shows the relationship between two numbers or measurements in the form of a grid. If this is a rectangular graph using Cartesian coordinate system, the two measurements will be arranged into two different lines at right angle to one another. One of these lines will be going up (the vertical axis). The other one will be going right (the horizontal axis).

Line Graph

A line graph refers to a kind of chart or graph that shows us information when a series of data is joined by a line. Similarly, it represents the changes in the data over a phase of time.

Bar Graphs

Bar graphs are one of the simplest one amongst the graphs. Bar graphs are the pictorial representation of data (generally grouped), in the form of vertical or horizontal rectangular bars, where the length of bars are proportional to the measure of data. They are also known as bar charts.

The bars drawn are of uniform width, and the variable quantity is represented on one of the axes. Also, the measure of the variable is depicted on the other axes. The heights or the lengths of the bars denote the value of the variable, and these graphs are also used to compare certain quantities. The frequency distribution tables can be easily represented using bar charts which simplify the calculations and understanding of data.

NOTES

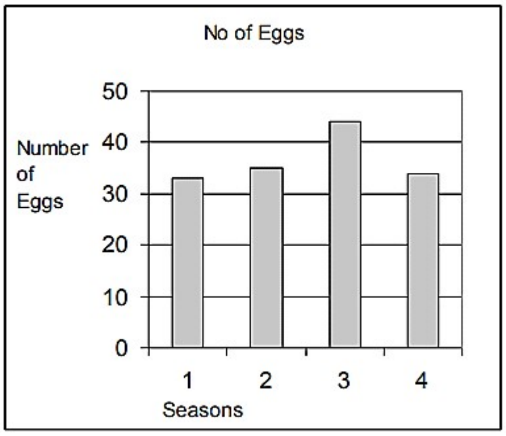
Aim 1: Construction of 'Bar Diagram' using biological data.

The following Table 1 gives the number of eggs laid by lizard in a season.

Table 1 *Number of Eggs Laid by Lizard in a Season*

Seasons	1	2	3	4
Number of Eggs	33	35	45	34

Solution: The required bar diagram is,



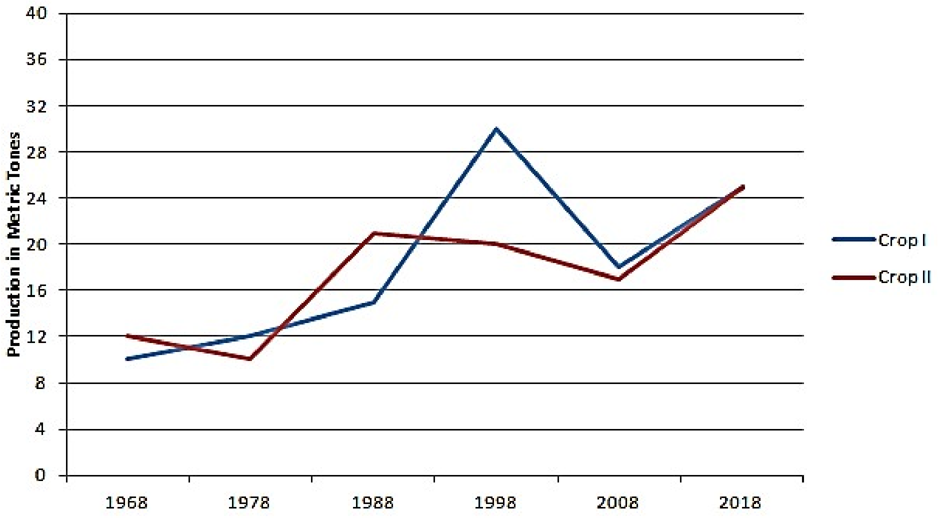
Aim 2: Construction of 'Line Graph' for the production of two types of crops for the given years.

The following Table 2 gives the production of two types of crops for the given years.

Table 2 *Production of Two Types of Crops for the Given Years*

	Production in Metric Tones	
Year	Crop I	Crop II
1968	10	12
1978	12	10
1988	15	21
1998	30	20
2008	18	17
2018	25	25

Solution: The required graph is,



3. CALCULATION OF MEAN, MEDIAN, MODE, VARIANCE, STANDARD DEVIATION AND STANDARD ERROR AND CHI-SQUARE TEST.

Statistics is a field of mathematics that pertains to data analysis. Statistical methods and equations can be applied to a data set in order to analyse and interpret results, explain variations in the data, or predict future data. Using the statistical information we can calculate:

- Average Value (Mean)
- Most Frequently Occurring Value (Mode)
- On Average, How Much Each Measurement Deviates from the Mean (Standard Deviation of the Mean)
- Span of Values Over Which Your Data Set Occurs (Range)
- Midpoint between the Lowest and Highest Value of the Set (Median)

Statistics is important in the field of engineering by it provides tools to analyse collected data. Using the statistical methods we can calculate the standard basic statistical functions, such as mean, median, mode, standard deviation of the mean, variance and standard deviations, correlation coefficients, Chi-square test, z-scores, p-values, etc.

Calculation of Averages or Central Tendency

The average in general term describes the centre of a series of measurements, like weight, height, or any other numerical features. The average or central value helps to compare it with similar type of observations on other groups and serves to

compare between two dissimilar series. There are three types of independent measures to determine central tendency, the mean, median and mode.

A. MEAN

Aim 1: Calculation of Mean.

The means are of three types, Arithmetic Mean, Geometric Mean and Harmonic Mean.

Arithmetic Mean: Arithmetic mean of the observed experiment scores is found by summing up all the observations and then dividing the total by number of observa-tions. The observation series is specified by the letter X, and the individual observations are specified by $X_1, X_2, X_3, \ldots\ldots\ldots\ldots\ldots, X_n$ and mean by (X). The total number of observations are specified by 'n' and the total sum of observation by 'Ó'.

Mean = Total of Σ of Observations / Total Number of Observations

Or, $X = X_1 + X_2 + X_3 — + X_n/n = \Sigma x_i/n$

This formula is appropriate for small size ungrouped series.

For example, if the number of oranges per plant is,

5, 6, 7, 8, 9, 5, 4, 5, 6, 7, 5, 5

Then,

$\Sigma X = (5 + 6 + 7 + \ldots\ldots\ldots\ldots + 5)$

$= 72$

Total Number of Observations 'n' = 12

Arithmetic Mean of Ungrouped Series,

$X = 72 / 12 = 6$

Geometric Mean: The geometric mean is the 'nth' root of products of 'n' items of a series. For example,

The geometric mean of items 4 and 25 will be $\sqrt{4} \times 25 = \sqrt{100} = 10$.

The equation for calculating the geometric mean is,

$$\sqrt[n]{x_1,\ x_2,\ x_3,\ x_4,\ \cdots, x_n} = \text{Anti log}\sqrt{\frac{\Sigma \log x}{n}}$$

$$\text{or, Antilog of } \frac{(\log x_1 + \log x_2 + \cdots\cdots + \log x_n)}{n}$$

Harmonic Mean: It is used for mea-surements on a reciprocal scale. The harmonic mean can be expressed as the reciprocal of the arithmetic mean of the reciprocals

of the given set of observations. As a simple example, the harmonic mean of 1, 4, and 4 is,

$$\left(\frac{1^{-1}+4^{-1}+4^{-1}}{3}\right)^{-1}=\frac{3}{\frac{1}{1}+\frac{1}{4}+\frac{1}{4}}=\frac{3}{1.5}=2$$

B. MEDIAN

Aim 2: Calculation of Median.

The median is the value separating the higher half from the lower half of a data sample, a population, or a probability distribution. For a data set, it may be considered as the 'Middle' value.

In general, for a set *x* of *n* elements, this can be written as:

$$\text{median}(x)=x_{(n+1)/2}$$

If the data set has an odd number of observations, then the middle one is selected. For example, consider the following list of seven numbers,

1, 3, 3, 6, 7, 8, 9

In this series, the median is the middle number 6, which is the fourth value.

For example, in the zoo list the different animals in the series are,

4, 6, 8, 10, 12, 14, 32

The median value is '10', which is an enhanced indicator method of average as compared to mean (86/7 = 12.3).

Median = (n + 1/2)th item, when total number of observations is 'n'.

Here (n + 1/2) is the observation (item) number and numerical values denotes the median.

C. MODE

The maximum repeated observations in the series is the modal number; on that basis the population may be unimodal, bimodal or multimodal. For example, in the series 5, 4, 3, 2, 2, 8, 7, 2, 9, 10, 11, the number 2 is repeated 3 times, whereas others once only. So modal value would be 2 and distribution of the population is unimodal.

D. VARIANCE AND STANDARD DEVIATION

In statistics, variance is the expectation of the squared deviation of a random variable from its mean. In other words, it measures how far a set of numbers is spread out from their average value.

The variance is the square of the standard deviation, the second central moment of a distribution, and the covariance of the random variable with itself. It is usually represented by σ^2, s^2, or Var (X).

The biological information, whether qualitative or quantitative, expressed in numerical number are very much variable. This variability is an essential feature of many types of materials. Such variability are of three types — biological, real and experimental.

Biological variability is the variation among individuals within the same group or category in respect to certain variety. Real variability is the difference between two readings or observations among the different classes more than defined limits in nature. Experimental variability is the difference developed due to sampling defects, or instrumental defects or measurement defects or personal defect.

Any type of deviation from the arithmetic mean is termed as variability. The sum of squared deviations from the arithmetic mean gives the total variance. Deviations from the arithmetic's if denoted by "di" which is equal to x_i-X.

Total variance formula is,

$$\text{variance} = \sum^{n} di^2 = \sum\left(x_i - \overline{X}\right)^2 \text{ where, } i = 1,$$

2,..., n = no. of observations.

Mean of deviations' square gives the variance of the sample. That is,

Variance $(s^2) = \Sigma di^2 / n - 1$

The divisor "n – 1" is termed as degrees of freedom (df). The degrees of freedom correspond to the number of independent deviations that are available from the data or can be calculated by deducting from the number of values available from the number of constants that are calculated from the data. Here mean is such a constant and hence the number of degrees of freedom is one less than n, the number of observations.

The standard deviation is a measure of the amount of variation or dispersion of a set of values. A low standard deviation indicates that the values tend to be close to the mean (also called the expected value) of the set, while a high standard deviation indicates that the values are spread out over a wider range.

Standard Deviation may be abbreviated SD, and is most commonly represented in mathematical texts and equations by the lower case Greek letter sigma 'σ', for the population standard deviation, or the Latin letter s, for the sample standard deviation.

Aim 3: Calculation of Mean, Variance and Standard Deviation.

The measurement of the heights of five dogs (in millimeters) is given below.

The heights (at the shoulders) are:

600mm, 470mm, 170mm, 430mm, 300mm.

Find out the Mean, the Variance, and the Standard Deviation.

Solution:

Mean = (600 + 470 + 170 + 430 + 300) / 5

= 1970 / 5

= 394

Therefore, the **mean (average) height is 394 mm.**

Now for each dog's calculate the difference from the Mean:

Dog 1 = 600 – 394 = 206

Dog 2 = 470 – 394 = 76

Dog 3 = 170 – 394 = –224

Dog 4 = 430 – 394 = 36

Dog 5 = 300 – 394 = –94

To calculate the Variance, take each difference, square it and then average the result as shown below:

Variance

σ^2 = $(206^2 + 76^2 + (“224)^2 + 36^2 + (“94)^2) / 5$

= (42436 + 5776 + 50176 + 1296 + 8836) / 5

= 108520 / 5

= 21704

So the **Variance is = 21,704.**

Standard Deviation

The Standard Deviation is the square root of Variance, therefore:

$\sigma = \sqrt{21704}$

= 147.32...

= 147 (to the nearest mm)

Therefore, the **Standard Deviation is 147mm.**

E. STANDARD ERROR

The standard deviation of the means is commonly termed as standard error and is equal to,

SE = SD/√n.

It is a quantity which can be directly calculated from standard deviation of the sample and the sample size.

Suppose the mean systolic blood pressure of 566 males is 128.8 mm and SD is 13.05 mm.

Then SE = SD/√n = 13.05/√566 = 0.55

Standard Error (SE) of Difference

A problem more commonly met with in agricultural and biological research is the comparison of two sample means. The standard error of difference of means of two samples of sizes n_1 and n_2 drawn from a population with,

standard deviation σ is $\frac{\sigma}{\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$ and that its

estimate is provided by $\frac{S}{\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$

$$\text{where } S = \sqrt{\frac{(n_1-1)S_1^2+(n_2-1)S_2^2}{n_1+n_2-2}}$$

S_1 and S_2 standing for the SD of two samples respectively.

$$\text{SE of difference} = \frac{S}{\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$$

Aim: To calculate the Standard Error (SE) of Difference.

In a nutritional study 100 children were conducted who were given a usual diet and Vitamin A and Vitamin D tablets. After 6 months their average weight was 30 kg with Standard Deviation of 2 kg, while the average weight of control group of 100 children with usual diet is 29 kg with Standard Deviation 1.8 kg. Is it possible that Vitamin A and Vitamin D tablets are responsible for this difference?

Solution: As per the formula,

$$S = \sqrt{\frac{(100-1)(2)^2 + (100-1)(1.8)^2}{100+100-2}}$$

$$= \sqrt{\frac{(99\times 4)+(99)(3.24)}{100+98}} = \sqrt{\frac{396+320.76}{198}}$$

$$= \sqrt{\frac{716.76}{198}} = \sqrt{3.62} = 1{\cdot}9.$$

$$\text{Then SE of difference} = \frac{1{\cdot}9}{\sqrt{\frac{1}{100}+\frac{1}{100}}}$$

$$= \frac{1{\cdot}9}{\sqrt{\frac{2}{100}}} = 1.9 \times 0.14 = 0.27.$$

The ratio of observed difference between means to Standard Error or SE of difference is Z.

Z = 30 – 29/0.27 = 1/0.27 = 3.7

Because the value of the ratio (Z) is 3.7 times to SE of differences, therefore the observed difference is highly significant. Consequently the Vitamin A and Vitamin D tablets have effect on weight gain.

The normal probability table indicates that when this proportion is more than 1.96 times, the observed deviation is significant at 5% level (P0.05). Similarly, in a deviation 2.58, its SE is significant at 1% (P0.01) level.

F. CHI-SQUARE TEST

The Chi-Squared Test, also written as $\chi 2$ test, is a statistical hypothesis test that is valid to perform when the test statistic is chi-squared distributed under the null hypothesis, specifically Pearson's Chi-squared test and variants thereof. Pearson's Chi-squared test is used to determine whether there is a statistically significant difference between the expected frequencies and the observed frequencies in one or more categories of a contingency table.

In the standard applications of this test, the observations are classified into mutually exclusive classes. If the null hypothesis that there are no differences between the classes in the population is true, the test statistic computed from the observations follows a $\chi 2$ frequency distribution. The purpose of the test is to evaluate how likely the observed frequencies would be assuming the null hypothesis (H_0) is true.

The Chi-squared test is specifically used in the biological and scientific analysis tests when the data fit into the YES or NO, or CHANGE or NO CHANGE categories. Whether some event did or did not have an effect, can be better known from this test. Additionally, the Chi-square test might be used in the evaluation of a drug, against fever or other cases in the investigation on the efficacy of a drug, one group of patients with fever are subjected to the drug, while the control group receives an impotent substitute.

The data at the end of observations are shown below in Table 1:

Table 1 *Number of Patients*

	Fever	Normal	Total	% Normal
Control	46	7	53	13.2%
Drug	12	31	43	72.2%
Total	58	38	96	39.6%

At the end of the treatment period, for the totals of the two groups 39.6% (38 × 96 × 100) of all the patients had no fever. In case there exists no difference (null hypothesis; H_0) between the two groups, then in the control group 21 patients (53 × 39.6%) would be normal and 32 patients (53 – 21) would still have fever.

The same procedure is carried for the drug group and the data may be arranged as shown in Table 2:

Table 2 *Number of Patients*

	Fever		Normal		Total
	Observed	**Expected**	**Observed**	**Expected**	
Control	46	32	7	21	53
Drug	12	26	31	17	43
Total	58	58	38	38	96

Therefore,

$$\chi 2 = \frac{(46-32)^2}{32} + \frac{(12-26)^2}{26} + \frac{(7-21)^2}{21} + \frac{(31-17)^2}{17}$$

$$= 6.32 + 7.55 + 9.34 + 11.52 = 34.73$$

Once degree of freedom is still there, as after calculation of one value the other three can be obtained by subtraction. As per the standard table of Chi-square values for degree of freedom, for one degree of freedom and Chi-square values 34.73, p is less than 0.001. That means the probability of difference is less than 1 in 1000.

Such a large difference between two groups under study can occur only by chance. From the highly significant difference between the two groups it may be assumed that the drug is effective in lowering fever.

4. IN SILICO ANALYSIS

In biology and other experimental sciences, an 'IN SILICO' experiment is one which is performed on computer or via computer simulation. The phrase is pseudo-Latin for 'IN SILICO', referring to silicon in computer chips. It was coined in 1987 as an allusion to the Latin phrases in vivo, in vitro, and in situ, which are commonly used in biology (especially systems biology). The latter phrases refer, respectively, to experiments done in living organisms, outside living organisms, and where they are found in nature.

IN SILICO study in medicine is thought to have the potential to speed the rate of discovery while reducing the need for expensive lab work and clinical trials. One way to achieve this is by producing and screening drug candidates more effectively. In 2010, for example, using the protein docking algorithm EADock, researchers found potential inhibitors to an enzyme associated with cancer activity IN SILICO. Fifty percent of the molecules were later shown to be active inhibitors in vitro. This approach differs from use of expensive High-Throughput Screening (HTS) robotic labs to physically test thousands of diverse compounds a day often with an expected hit rate on the order of 1% or less with still fewer expected to be real leads following further testing.

As an example, the technique was utilized for a drug repurposing study in order to search for potential cures for COVID-19 (SARS-CoV-2).

Cell Models

The cellular behaviour of IN SILICO computer models can be established. For example, in 2007 researchers developed an IN SILICO model of tuberculosis to aid in drug discovery, with the prime benefit of its being faster than real time simulated growth rates, allowing phenomena of interest to be observed in minutes rather than months.

Limitations in the understanding of molecular dynamics and cell biology as well as the absence of available computer processing power force large simplifying assumptions that constrain the usefulness of present IN SILICO cell models, which are very important for IN SILICO cancer research.

Genetics

Digital genetic sequences obtained from DNA sequencing may be stored in sequence databases, be analysed, be digitally altered or be used as templates for creating new actual DNA using artificial gene synthesis.

IN SILICO computer-based modeling technologies have also been applied in:

- Whole cell analysis of prokaryotic and eukaryotic hosts, for example *E. coli*, *B. subtilis*, Yeast, CHO- or Human Cell Lines.

- Discovery of potential cure for COVID-19.
- Bioprocess development and optimization, for example optimization of product yields.
- Simulation of oncological clinical trials exploiting grid computing infrastructures, such as the European Grid Infrastructure, for improving the performance and effectiveness of the simulations.
- Analysis, interpretation and visualization of heterologous data sets from various sources, for example genome, transcriptome or proteome data.
- Protein design. One example is RosettaDesign, a software package under development and free for academic use.

Protocol for IN SILICO Gene-Expression Profiling

Gene-expression profiling is a powerful technique for studying biological processes at the molecular level. Gene activity or expression, can be assessed by protein identification but gene expression is usually investigated by examining the RNA message or transcript. Two high-throughput methods that are commonly used for comprehensive gene expression profiling are RNA sequencing with Next-Generation Sequencing (NGS) and DNA microarrays.

For the modern biologist, there are numerous computational strategies that can be employed to assay gene expression. Many of these are based on utilising collections of Expressed Sequence Tags (ESTs), unique segments of cDNA with base sequences identical to at least part of the coding region of a gene. Because a large number of ESTs from diverse organ-derived and disease-derived cDNA libraries are being deposited in different databases, EST libraries are therefore an ideal source for expression profiling since EST clone frequency is in principle, proportional to the corresponding gene's expression level in a given tissue.

Usually analysis of a protein, which includes characteristics as well as determination of structure, can be done IN SILICO which is obtainable through the use of bioinformatics tools and an array of various online databases.

IN SILICO Protein-Protein Interactions (PPIs)

Protein-Protein Interactions (PPIs) are useful for understanding the signalling cascades, predicting protein function, associating proteins with disease and fathoming drug mechanism of action. Currently, only <"10% of human PPIs may be known, and about one-third of human proteins have no known interactions. Protein-Protein Interactions (PPIs) underlie the molecular mechanisms of most biological processes. Mitogen-Activated Protein Kinases (MAPKs) can be dephosphorylated by MAPK-specific phosphatases, such as

PP2C, which are critical to transduce extracellular signals into adaptive and programmed responses.

Homologous proteins are identified and then compared in regard to their structural and functional properties to know the unfamiliar ones. Such data can then be used in laboratory experiments to establish properties and subsequently lead to discover novel proteins (Kallberg, 2002). The verifications can be used within bioinformatics so as to attain much more accurate and detailed results in terms of function and Protein-Protein Interaction or PPI. Hence, IN SILICO methods play a significant role and need to be used in collaboration with biology, biochemistry, medicine and so on.